Celiac disease
Towards new therapeutic modalities

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Aan mijn familie

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Chapter 1

Celiac disease
General Introduction and Thesis Outline

Celiac disease

GENERAL INTRODUCTION

Celiac disease, originally thought to occur only rarely in childhood, is now recognized as a common condition that might be diagnosed at any age. Due to large screening programs it is now well established that between 0.5 and 2% of the general population in the Western Hemisphere suffers from celiac disease, making celiac disease one of the most common food induced diseases (1,2).

The disorder was previously called celiac sprue, based on the similarity with tropical sprue that is characterized by diarrhea, emacination, stomatitis and malabsorbtion (3). It is now known that celiac disease is a genetically–predisposed disease precipitated by ingestion of gluten, the major storage proteins in wheat, and similar proteins in related cereals like barley, rye and triticale (hybrid between wheat and rye) (4). The proximal small intestine is the common localization point of celiac disease related lesions, but in some individuals the entire small intestine is involved. As a result, malabsorbtion of iron, folic acid, calcium and fat-soluble vitamins occurs, which causes iron and folate deficiency and reduced bone density. Diarrhea is the classical sign of celiac disease (5,6). It usually appears when the disease has progressed to the distal bowel and is accompanied by abdominal pain and discomfort. Next to diarrhea, abdominal distention and failure to thrive often occur in infants and young children with the disease while extra-intestinal manifestations as short stature, neurological symptoms or anemia are also encountered. Less common complications include constipation, weight loss, dermatitis herpetiformis, hypoproteinemia and elevated liver enzyme levels.

For a positive diagnosis of celiac disease a histological examination of a small bowel biopsy and a clinical improvement upon the introduction of a gluten-free diet is required. Typically, the examination of the small intestinal biopsy reveals villous atrophy, crypt hyperplasia and intraepithelial lymphocyte infiltration. Disease severity is related to the extent of intestinal damage and scored conform the Marsh classification (7). In the past, a second biopsy would be taken after a period of gluten-free diet, when the small intestinal mucosa is expected to a have a normal histology and a third one after a gluten challenge to demonstrate that this led to deterioration. In practice the second and third biopsies are not always taken as the characteristic morphological changes in combination with the disappearance of symptoms following a gluten-free diet is considered sufficient proof for a positive diagnostic. Moreover, less invasive diagnostic methods have become available that may lead to a further reduction in the taking of biopsies. These serological assays measure the presence and titer of IgA antibodies specific for gliadin, deamidated gliadin, endomysium and tissue transglutaminase (8,9). Several studies have been performed to test the sensitivity and specificity of these tests. While the assays measuring gliadin specific antibodies are no longer

considered sensitive or specific enough to be useful in the diagnosis of celiac disease, the assays measuring antibodies against deamidated gliadin, endomysium and anti-tissue transglutaminase (tTg) are considered highly sensitive and specific and therefore widely used in the diagnosis (10,11).

Next to patients with "classical" celiac disease, many patients have "silent" celiac disease. Such patients have no or only mild symptoms and truly asymptomatic individuals are usually identified only after screening of high-risk groups. At the other end of the spectrum are patients with refractory celiac disease (RCD) (12). In such patients intestinal inflammation persists despite a gluten-free diet, see below.

Thus, there is a large spectrum of symptoms and complications that can manifest, but it is largely unknown why this can differ so much between patients.

The disappearance of symptoms upon the introduction of a gluten-free diet is expected. Recurrent or new symptoms should raise questions. The patients may intentionally or non-intentionally ingest gluten which can be solved by closely monitoring their diet. Alternatively, and more seriously, a complication has developed like intestinal adenocarcinoma or RCD. Two types of RCD can be distinguished: RCD type I, with a normal intraepithealial lymphocyte population, and RCD type II, characterized by an aberrant intraepithelial lymphocyte population. RCD type II can culminate in the development of enteropathy associated T cell lymphoma (EATL), a potentially fatal complication. Treatment of RCD involves nutritional support together with a strict gluten-free diet. The use of corticosteroids can induce clinical improvement. Treatment of EATL includes chemotherapy, surgical removal and stem-cell transplantation but this is often insufficient.

In general, patients with celiac disease have an overall higher risk of cancers compared to the general population. Next to EATL, reported cancers include T-cell and B-cell non Hodgkin's lymphomas, oropharyngeal and esophageal adenocarcinoma and cancers of the small and large intestine, hepatobiliary system and pancreas (13,14). Celiac disease patients also have an increased risk to develop autoimmune diseases, partly due to the influence of the disease predisposing HLA-molecules (see below) and partly due to other immune related genetic variants that influence the risk of disease development.

Celiac disease: immunological aspects

Almost without exception celiac disease develops only in genetically predisposed individuals: over 98% of the patients express either HLA-DQ2 or HLA-DQ8. The expression of these molecules is thus required but not sufficient for disease development as the majority of HLA-DQ2 and HLA-DQ8 individuals will never develop celiac disease. In addition to HLA-DQ, several other non-HLA genes have been identified that influence the likelihood of disease development but their influence is much smaller as that of HLA-DQ and the molecular basis for the association remains largely elusive (15-17).

In contrast, the association of the disease with HLA-DQ became clear after the observation that gluten-specific, HLA-DQ-restricted T cell clones could be isolated from biopsies of celiac disease patients while such cells were not found in healthy controls (18). Further research revealed that these T cells respond to gluten peptides formed as

the result of the activity of proteases present in the gastro-intestinal tract. Moreover, it was observed that these peptides are modified by enzyme TG2, which can convert the amino acid glutamine in gluten peptides into the negatively charged glutamic acid. Due to this introduction of negative charge(s) into gluten peptides they can bind with high affinity to HLA-DQ2 or HLA-DQ8 and trigger (stronger) T cell responses (19,20). TG2, one of the main auto antigens involved in the disease (21), is normally present in an inactive form in the intracellular environment and released upon mechanical or inflammatory stress. The release of TG2 is thus a critical factor in disease development. The factors that determine and/or regulate this release are currently unknown.

Next to their capacity to trigger adaptive T cell responses, it is also claimed that gluten can induce innate immune responses (22-24). The mechanism, through which this is mediated, however, remains obscure.

Gluten and gluten-like molecules: the causative agent

Gluten (also known as prolamins) is a complex mixture of water-insoluble proteins routinely produced by washing dough from white flour with water. The resulting fraction comprises about 70% protein with the rest being primarily starch and lipids.

Gluten is classically divided into the alcohol-soluble gliadin fraction and the alcohol insoluble glutenin fraction (25). Both contain multiple proteins which can be further separated by electrophoresis. The gliadins consist of monomeric proteins, which are classified into α-, β-, γ- and ω-gliadins. The glutenins consists of polymeric proteins stabilized by disulfide bonds. These bonds need to be reduced before the component subunits can be separated into 2 groups, the high molecular weight (HMW) and the low molecular weight (LMW) subunits.

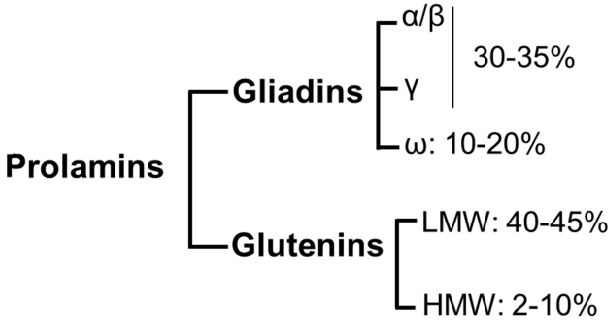

Figure 1. Distribution of different prolamin fractions wheat.

Gluten proteins have characteristic properties. They contain a very high amount of the amino acids glutamine (~ 30%, hence the name) and proline (~ 17%). Due to the latter gluten is partially resistant to degradation in the gastrointestinal tract so that large fragments survive long enough to bind to the disease predisposing HLA-DQ molecules. Moreover, due to the glutamine content, the enzyme TG2 can convert multiple

glutamines in gluten proteins into glutamic acid, generating high affinity ligands for HLA-DQ2 or HLA-DQ8. Consequently, multiple immune stimulatory peptides can be found in any gluten protein. Moreover, every class of gluten molecules has been found to encode such peptides (26-28). Gluten thus encodes a large array of immune stimulatory peptides and this is likely linked to its disease inducing properties.

Next to wheat also other cereals like barley, rye and triticale are known to be toxic for celiac disease patients (29). Wheat, barley and rye belong to the same Triticeae family, which explains the similarities observed between the gluten proteins from wheat and the analogous proteins in the other cereals (Figure 2). Not surprisingly, gluten specific T cells can recognize homologous sequences from wheat, barley and rye (29).

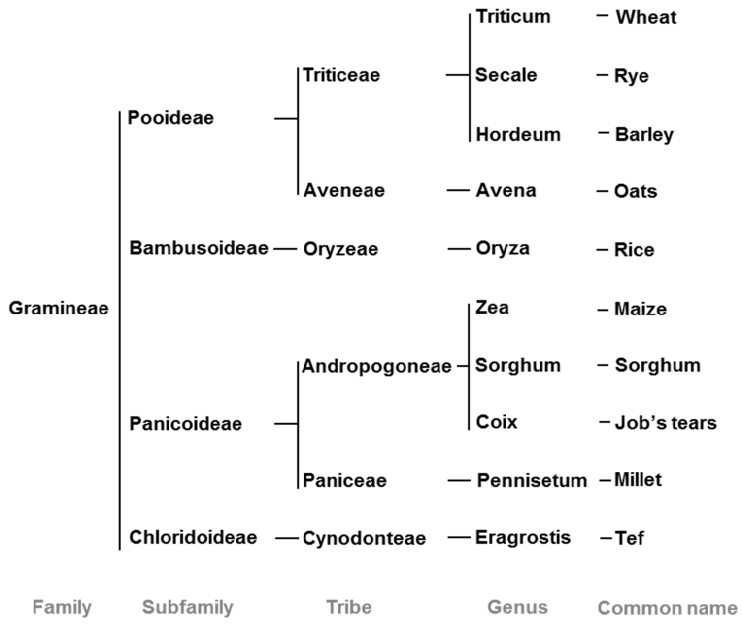

Figure 2. Classification of Gramineae plant family.
Adapted from M. Kagnof, J. Clin. Ivest. 117:41-49, 2007.

Oats are phylogenetically more distantly related to wheat than barley and rye (Figure 2) and non-contaminated ('pure') oat is considered as gluten-free in EC-regulation 41/2009. Although several studies show that more than 99% of celiac disease patients can safely consume oats, care should be taken as two studies clearly demonstrated that oats contains gluten like epitopes that can be recognized by patient derived T cells (29,30). An explanation for this apparently contradiction is in the key differences between the avenins of oat and the gluten and gluten-like molecules in the other cereals. First, the amount of avenins in oat grains is substantially lower than that of gliadin in wheat (10% of the total protein content in oat, compared to 40-50% in

wheat), as most storage proteins in oat are globulins. Second, avenins contain less proline, the amino acid that contributes especially to the resistance of gluten to degradation in the gastrointestinal tract and that is crucial for the specific modification by the enzyme tTG that is linked to gluten toxicity. As a result a lower amount of stimulatory peptides are released after oat digestion.

There are other alternative cereals that are safe for consumption by celiac disease patients, all distantly related to wheat (Figure 2). Originally form Ethiopia, Teff is used to make a flat bread named *injera*. Extensive research on a selection of Teff varieties demonstrated that Teff protein extracts are not recognized by gluten specific T cells and antibodies (31). Furthermore, Teff is consumed by a large number of Dutch celiac disease patients without causing any symptoms (32). In addition, it is well established that rice and maize are safe cereals that can be introduced in the gluten-free diet adding nutritional value. Moreover, sorghum and millet, more common in Africa, and Job's tears, cultivated in East Asia, are also considered safe for celiac patients. As all these cereals are distantly related to wheat, their prolamin proteins are unlike those of wheat, barley and rye, hence they lack immune stimulatory sequences and are safe for consumption by celiac disease patients.

Thus, the knowledge about gluten structure and phylogenetic origin helps us to understand why some cereals are toxic and others not, laying a sound basis for improvements in the gluten-free diet.

Detection of gluten

The only acceptable treatment for celiac disease is a gluten-free diet, but accidental gluten ingestion often happens. Contamination of non-toxic cereals with wheat, barley or rye due to co-culture or during transport, storage and/or industrial processing and the use of gluten as a hidden ingredient to improve the properties of different food products cause accidental ingestion. It is well established that commercially available oats is almost invariably contaminated with other cereals, excluding the use of such oats in a gluten-free diet (33,34).

Exposure of patients to gluten prevents healing of gut mucosa, reactivation of specific T cells and reappearance of symptoms. Although not every patient is equally sensitive to gluten exposure, it was reported that exposure to 1 mg of gluten prevented mucosal recovery (35). Therefore, to safeguard patients from gluten exposure, sensitive methods for gluten detection are required and have been developed.

Three techniques have been explored. Monoclonal antibodies raised specifically against gluten are most commonly used. These antibodies detect either known toxic gluten sequences (36-38) or highly repetitive sequences like QQPFP from gluten (39). Some of these antibodies have been incorporated in ELISA-immunoassays that can be used for detection of gluten contamination in food (40-43).

An alternative approach is the use of Real Time PCR-techniques (34,44) using primes encoding repetitive sequences from gliadin.

The third possibility is the use of mass spectrometry techniques (45). The procedure is based on the determination of characteristic gliadin mass spectra and can be

applied for screening of both processed and unprocessed gluten containing food samples.

In general, natural gluten-free foods should not contain over 20 ppm of gluten while foods rendered gluten-free should not contain over 100 ppm in order to be suitable for use in a gluten-free diet. Consumption by celiac disease patients of products containing more than 100 ppm gluten was associated with appearance of symptoms.

Treatment

The current treatment for celiac disease is strict adherence to a life-long gluten-free diet. The wide-spread use of gluten and gluten-derived starch in the food industry makes the gluten-free diet challenging. It is not surprising that a considerable proportion of patients, especially adolescents, are interested in alternative treatments that would allow gluten consumption.

One approach would be blocking the binding site in HLA-DQ2 and HLA-DQ8 to prevent the presentation of disease-inducing gluten peptides to the disease associated gluten specific T cells. Several groups have now designed peptide based compound that exhibit high affinity for the HLA-DQ2 binding site (20, 21, 22). It remains to be established if such compounds are indeed capable of inhibiting gluten specific T cell responses.

Alternatively, compounds that would block tissue transglutaminase could be used to treat patients as tissue transglutaminase plays a central role in the formation of many gluten epitopes through deamidation (46). Thus, although T cells have been described that recognize non-deamidated gluten epitopes, blocking of tissue transglutaminase would prevent a substantial part of the gluten specific T cell response. Such blockers, however, are expected to exhibit side effects, like impaired wound healing, limiting the applicability of this approach.

The perhaps most promising approach is the use of exogenous enzymes to improve gluten degradation in the gastrointestinal tract. It has been proposed that oral administration of a prolyl endoprotease might counteract the damaging effects of moderate amounts of gluten. Prolyl endoproteases can cleave the proline rich immunostimulatory gluten fragments, creating in this way new cutting sites for the enzymes residing in the gastro-intestinal tract. Several enzymes have been proposed, either in single (47,48) (49-51) or in combination therapies (52,53) and their efficiency was proved *in vitro*, *in vivo* (rats) and in *ex vivo* (T cell cultures) experiments. If these enzymes are indeed capable of degrading gluten proteins efficiently in the gastrointestinal tract remains to be determined.

Finally, the development of grains that contain no or low toxic gluten proteins, the holy grain, is an attractive alternative. Potentially, such grains can be developed by breeding diploid wheat varieties, incorporation of non-toxic gluten genes into harmless cereals such as rice, small RNA interfering technology or mutation of genes encoding the immunostimulatory sequences. Although such approaches are likely to prove to be a challenge, the existence of such a grain could form the base for a nutritionally improved diet for celiac disease patients.

THESIS OUTLINE

My project has focused on the development of alternatives to the gluten-free diet. Two different approaches were investigated: the use of enzymatic supplementation and the identification and/or development of a less/non-toxic cereal.

In **chapter 2** the characterization of monoclonal antibodies raised against T cell stimulatory gluten peptides is described. Their reactivity against the prolamins from wheat, barley, rye and oats was determined and compared with that of gluten reactive T cells. The results demonstrate that the antibody and T cell reactivity patterns overlap significantly, indicating that the antibodies can be used to detect toxic sequences in gluten. Subsequently, these antibodies were used in the studies aimed at the development of alternative to the gluten-free diet.

In **chapter 3** we propose a new strategy to generate non-toxic gluten. Our experiments demonstrated that non-immunogenic epitope variants were present in certain diploid wheat varieties that differ one amino acid with the toxic variant. Moreover, we found that by the introduction of this naturally occurring amino acid substitution in other toxic epitopes their T cell stimulatory activity was likewise eliminated. This approach can thus be used to generate gluten genes that are devoid of any T cell stimulatory activity and presumably safe for consumption by celiac disease patients.

In **chapter 4** we investigate the safety of oats for consumption by celiac disease patients. We confirmed that commercially available oats are without exception contaminated with other cereals. Perhaps more importantly, we demonstrate that contamination-free oat varieties differ in their capacity to stimulate an avenin-sensitive gamma-gliadin specific T cell line derived from a patient with CD, opening the way to select and/or breed oats varieties that contain no harmful gluten-like proteins.

In **chapter 5** we investigate the potential of AN-PEP, a prolyl-endoprotease produced by the microorganism *Aspergillus niger*, to degrade gluten in an artificial gastrointestinal tract system. The enzyme proved very efficient in degrading all toxic epitopes in this system, even when a complex meal was introduced. These “in vitro” studies now justify a clinical trial to assess the safety and effectiveness of the enzyme for gluten degradation in patients.

In **chapter 6** I discuss how the results may lead to novel treatment modalities and novel foods in the near future.

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Chapter 2

Fine specificity of monoclonal antibodies against celiac disease-inducing peptides present in the gluteome

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ABSTRACT

In Celiac Disease (CD) patients peptides, derived from dietary gluten, are recognized by HLA-DQ2 restricted $CD4^+$ T cells which results in inflammation. Such immune-stimulatory peptides are found in both gliadins and glutenins. Monoclonal antibodies (mAb) against these peptides can be used to screen food for the presence of such peptides. We aimed to determine specificity of five mAb raised against T cell stimulatory peptides found in α- and γ-gliadins and low and high molecular weight glutenins and to compare it this with the specificity of patient-derived T cells. Moreover, to determine the suitability of these mAb for the detection of homologous proteins in barley, rye and oat. The reactivity of the mAb with gluten peptides, enzymatic gluten digests and intact gluten proteins was determined and compared with that of gluten-specific T cells using a combination of immunological and biochemical techniques. Furthermore, the reactivity of the mAb with gluten homologues in barley, rye and oat was determined. The specificity of the mAb largely overlaps with that of gluten-specific T cells. Moreover, the mAb detect several homologous peptides present in gluten proteins. All except the LMW-specific mAb also detect storage proteins present in barley and rye, while the γ-gliadin-specific mAb also recognises oat proteins. The mAb raised against T cell stimulatory peptides in gliadins and glutenins allow a comprehensive screen for the presence of harmful gluten and gluten-like proteins and peptides in food products. They can thus be used to guarantee the safety of food for CD patients.

INTRODUCTION

Celiac disease (CD) is caused by intolerance to gluten, storage proteins found in wheat. Typical symptoms are chronic diarrhoea or constipation, malnutrition, anaemia, fatigue, growth retardation and migraine. These symptoms are the result of an inflammatory process that causes (sub) total villous atrophy in the small intestine which decreases the normal uptake of nutrients from food. With an incidence of 0.5-1.0%, CD is the most common immune-mediated food intolerance in the Western world.

During digestion, the gluten proteins are enzymatically broken down in the gastrointestinal tract. However, because of the high proline content of the gluten proteins, the degradation is not efficient and relatively large gluten peptides persist (1,2). In CD patients such gluten peptides, when bound to the disease-predisposing HLA-DQ2 or HLA-DQ8 molecules, can trigger a CD4+ T cell mediated inflammatory immune response(3-5). The gluten-derived peptides that trigger the inflammatory CD4+ T cell responses originate from proline- and glutamine-rich regions of α- and γ-gliadins and from the low (LMW) and high molecular weight (HMW) glutenins (6-8). Next to wheat also other cereals like barley, rye and oat contain storage proteins, the hordeins, secalins and avenins respectively. These related storage proteins can also trigger the immune system of CD patients (9). The collection of all gluten and gluten-like proteins is referred to as the gluteome (10).

A strict lifelong gluten-free diet is currently the only available treatment for CD. This diet is complicated by the fact that gluten is extensively used in the food industry and, as a result, can be found in many products that are not normally associated with gluten or wheat, including sauces, sweets and medication. According to the guidelines of the Codex Alimentarius gluten-free food should contain less than 200 ppm gluten when rendered gluten-free and less than 20 ppm when naturally gluten-free, as mentioned in the Report of the 25th Session of the Codex Alimenatrius Committee on Nutrition and Foods For Special Dietary Uses, Bonn 2003. Most likely the acceptable level for rendered gluten-free foods will be reduced in the near future as in a new draft for the standards by the Codex Alimentarius a maximum level of 100 ppm is recommended (//ftp.fao.org/codex/ alinorm07/al30_26e.pdf). To determine if foods intended for the gluten-free market meet these strict requirements sensitive assays for gluten detection are required. The detection of gluten in food is complicated as gluten is a large family of proteins that is divided into two biochemical distinct subfamilies, the glutenins and gliadins. The former can be further subdivided into the LMW- and the HWM-glutenins while the latter can be further subdivided into α-, γ- and ω-gliadins, and each of these groups consists of a mixture of highly similar but distinct proteins (11). Another factor complicating the detection of gluten is that both intact gluten proteins and gluten hydrolysates are used in the food industry. This means that, ideally, gluten detection assays should be able to detect both intact gliadins and glutenins and fragments thereof. Moreover, since the disease-related T cell stimulatory peptides of gluten have been identified, it would be a major advantage if such gluten detection assays

would specifically detect these peptides rather than indicate a level of overall gluten content.

In order to develop a robust assay that would meet these requirements we have developed mAbs against T cell stimulatory peptides present in α- gliadin (Glia-α9, Glia-α20 epitopes), γ-gliadin (Glia-γ1 epitope), LMW-glutenin (Glt156) and HMW-glutenin. Some of these antibodies have already been used to develop highly sensitive competition assays for the detection of proteins and peptides that harbour these sequences(12-14).

In the present study, an extensive characterization of the specificity of these mAb was carried out to determine their usefulness for the detection of various gluten and gluten-like peptides present in cereals that are implicated in CD.

MATERIALS AND METHODS

Generation of monoclonal antibodies

For the generation of mAb Balb/c mice were immunized with synthetic peptides corresponding to known T cell stimulatory epitopes that were coupled to tetanus toxoid (Table 1). Fusion, purification and screening of the hybridomas were performed as described previously (12-14).

Database searches

Searches were performed in the UniProt kB database for variants of the the Glia-α9, Glia-α20, Glia-γ1, LMW- and HMW-glutenin gluten epitopes using FASTA alignment as described (15). The following sequences were used in the searches: Glia-α9: QLQPFPQPQLPY/E; Glia-α20: PFRPQQPYPQPQPQ; Glia-γ1: QQSFPQQQRPFIQPSL; LMW: PPFSQQQQSPFS. For the variants of the HMW epitope previously described results were used (7). Variants of the Glia- α2, Glia-α9, Glia-α20, Glia-γ1 epitopes present in gluten-like proteins from barley, rye and oat were identified previously (15).

Synthetic peptides

Peptides were synthesized by standard Fmoc chemistry on a SyroII peptide synthesizer as described previously. The integrity of the peptides was checked by rpHPLC and mass spectrometry. When required, Biotin was introduced in the resin-bound peptides by a two hour coupling with a 6-fold equimolar preactivating mixture of biotin and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop).

Peptide recognition assay

Elisa plates (Nunc Maxisorb immunoplate, Nunc, Copenhagen, Denmark) were coated overnight (100 µl/well) at room temperature with the various antibodies in a concentration of 2-5 µg/ml in phosphate buffered saline (PBS, 154 mM NaCl, 1.4 mM Na_2HPO_4/NaH_2PO_4 pH 7.4). The plates were washed with PBS/0.02% (w/v) Tween-20 and the residual binding sites on the plates were blocked by incubation for 30 minutes at room temperature with 150 µl/well 2% skim milk in PBS (Fluka, Zwijndrecht, The

Netherlands). After a washing step, the plates were incubated for 1 hour with the biotinylated peptides in a concentration range of 0.5-2 µg/ml in PBS/0.2% Tween-20/ 0.2% skim milk. After this step, the plates were washed and incubated for 30 minutes with an excess of streptavidin-HRP (Sigma Aldrich, Zwijndrecht, The Netherlands). After a washing step, bound peroxidase was visualised by incubation with a solution of 3,3',5,5'-tetramethylbenzidine (TMB, Sigma Aldrich). The colour reaction was stopped by the addition of 100 µl/well 2M H_2SO_4. Finally, absorbance was read on a multiscan plate reader (Wallac, Turku, Finland).

Direct binding assay

Direct binding assays were, in general, performed like the peptide binding assay. In short, Elisa plates (Nunc Maxisorb immunoplate, Nunc,Copenhagen, Denmark) were coated overnight at room temperature with the various peptides in a concentration range of 10-1,25 µg/ml in PBS, 100 µl/well. The plates were washed and the residual binding sites were blocked. After a washing step, the plates were incubated for 1 hour with the various mAb at a concentration of 1,5 µg/ml in PBS/0.2% Tween-20/ 0.2% skim milk. After this step, the plates were washed and incubated for 30 minutes with an excess of rat-anti-mouse, HRP conjugated polyclonal antibodies (Sigma Aldrich). After a washing step, the assay was stained and absorbance was read as described above.

Immune precipitation assay

For immune precipitation, a pepsin/ trypsin digest of wheat flour was incubated with the various monoclonal antibodies covalently coupled to sepharose beads (2-3 mg/ml). After 120 minutes of gentle mixing at 4°C, the beads were washed sequential with 120 mM NaCl, 20 mM Tris HCl pH 8.0; 1 M NaCl; 20mM Tris HCl pH 8.0; 20 mM Tris HCl pH 8.0 and 10 mM Tris HCl pH 8.0. Bound peptides were eluted with 10 ml 10% (v/v) acetic acid and analysed by mass spectrometry followed by database searches as described previously (10).

T cell proliferation assays

Proliferation assays were performed in triplicate in 150 µl RPMI-1640 (Gibco) supplemented with 10% human serum in 96-well flat bottom plates (Falcon) using 2 x 10^4 gluten-specific T cells stimulated with 10^5 irradiated HLA-DQ2-matched allogenic PBMCs (3000 RAD) in the presence of 1 µg peptides. All the peptides were deamidated by tissue transglutaminase (tTg) (BioTec, GmbH) in the presence of $CaCl_2$, overnight at 37° C. After 48 hours at 37° C, cultures were pulsed with 0.5 µCi of ^{3}H-thymidine, harvested 18 hours later and the ^{3}H-thymidine incorporation was quantified with a liquid scintillation counter.

Protein analysis by 1D SDS-PAGE and Western blotting

SDS-PAGE (12.5 % acrylamide gel) was performed under standard conditions. After separation proteins were either stained with Imperial™ Protein Stain (Pierce, Rockford IL, USA) or transferred to Polyvinylidene Difluoride (PVDF) membranes (Bio-Rad, Her-

cules CA, USA). For Western blot the proteins were visualized with mAbs specific for stimulatory T cell epitopes from α- and γ-gliadin, LMW- and HMW-glutenin (12-14).

In gel digestion of proteins

The desired gel bands, isolated from a Coomassie stained gel, were digested with chymotrypsin using the Proteineer DP digestion robot (Bruker, Bremen, Germany). The protocol supplied by the manufacturer was followed. Digested proteins were analyzed by mass spectrometry as described previously (10).

RESULTS

Generation of monoclonal antibodies specific for immune-stimulatory gluten sequences

For the development of reagents that detect immune-stimulatory gluten peptides mAb were raised against synthetic peptides corresponding to T cell stimulatory sequences present in gliadin (Glia-α9, Glia-α20, Glia-γ1), LMW- (glt-156) and HMW-glutenin (Table 1). The minimal amino acid sequences recognized by the mAb specific for the

Table 1. Overview of T cell stimulatory peptides used for the generation of gliadin and glutenin specific mAbs.

Protein	T cell stimulatory epitope	Immunisation peptide
α/β-gliadin	Glia-α9[3]	QPFPQPQLPYP
	Glia-α20	PFRPQQPYPQP
γ-gliadin	Glia-γ1	PQQSFPQQQRPFIQPSL
LMW-glutenin[1]	Glt-156[4]	PPFSQQQQSPFS
		PGQGQ(Q/P)GYYPTS(L/Q)QQP
HMW-glutenin[2]	HMW-glt	QGQQGYYPTSPQQ(P/S)

For the generation of mAbs Balb/c mice were immunized with synthetic peptides, containing the amino acid sequence of the T cell stimulatory epitopes coupled to tetanus toxoid. The minimal amino acid sequence recognized by gluten specific T cells is underlined.

[1] *LMW=low molecular weight*
[2] *HMW=high molecular weight*
[3] *Glia= gliadin*
[4] *glt= glutenin*

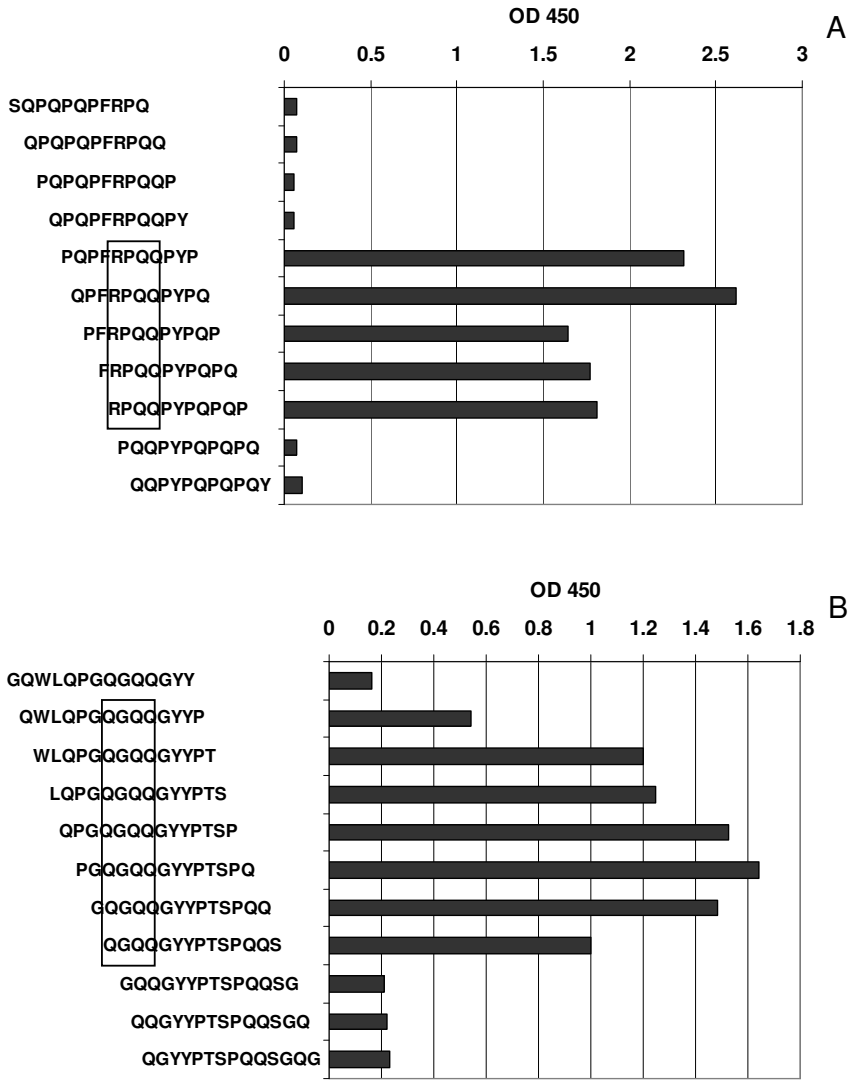

Figure 1. Minimal epitope recognized by mAb raised against T cell stimulatory epitopes of gliadin and glutenin.

The minimal epitopes recognized by the mAb was determined in a peptide recognition assay by testing their reactivity against a set of overlapping peptides. The sequence recognized by the mAb is indicated in boxes. (A) anti-Glia-α20 mAb (B) anti-HMW- glt mAb.

Glia-α9 and the Glia-γ1 epitopes were determined previously (13). With the use of a set of partially overlapping synthetic peptides the minimal amino acid sequences recognized by the mAb specific for the Glia-α20, LMW- and HMW-glutenin peptides were determined. Representative results are shown in Figure 1 while an overview of all results is given in Table 2. The results indicate that the sequence recognized by the mAb specific for the Glia-α9, Glia-α20 and HMW-glutenin peptides largely overlap with the T cell stimulatory sequences (Table 2). In contrast, the mAb specific for the Glia-γ1 and LMW-glutenin epitopes recognize sequences that (partially) overlap with either the C- or N-terminus of the T cell epitopes (Table 2).

Table 2. Comparison of minimal amino acid sequences recognized by gluten specific mAb and known T cell stimulatory gluten sequences.

epitope	*T cell*	*mAb* [4]
Glia-α9[1]	QLQPFPQPQLPY	QL**QPFPQPQ**LPY
Glia-α20	PFRPQQPYPQPQPQ	PF**RPQQPYP**QPQPQ
Glia-γ1	QPQQPQQSFPQQQRPFI	QPQQPQQSFPQ**QQRPFI**
LMW-glt[2]	QPPFSQQQQSPFSQ	Q**PPFSQQ**QQSPFSQ (LMW-1) QPPFSQQQ**QSPF**SQ (LMW-2)
HMW-glt[3]	QGQQGYYPTSPQQSG	**QGQQGYYP**TSPQQSG

The minimal amino acid sequence recognized by human T cells is underlined and the mAb epitope is in bold.
[1] *Glia=gliadin*
[2] *LMW- glt=low molecular weight glutenin*
[3] *HMW-glt=high molecular weight glutenin*
[4] *mAb=monoclonal antibody*

As many gluten proteins share a high degree of homology, we determined next if the mAb reacted specifically only with the peptide used for immunization or if they also detected the other T cell stimulatory sequences. The results (Figure 2A, 2B, 2D, 2E) indicate that the mAb specific for the Glia-α9, Glia-γ1, LMW- and HMW-glutenin peptides are highly specific for the peptide used for immunization. In contrast, the

Glia-α20 specific mAb also reacted with the Glia-α9 and Glia-γ1 peptides (Figure 2C). While the reactivity of this mAb with the Glia-α9 peptide is likely based on the shared sequence PQXPY between the Glia-α20 and Glia-α9 peptides, the basis for the reactivity with the Glia-γ1 peptide is less clear.

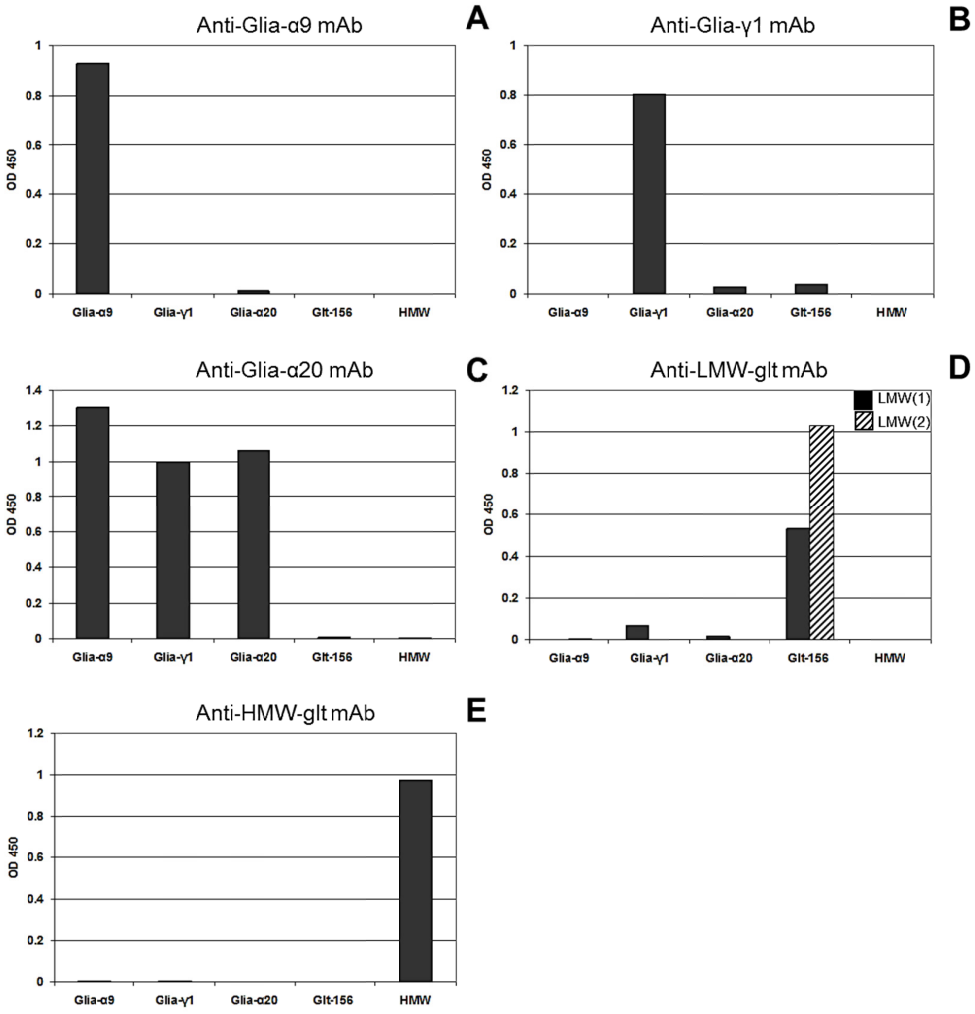

Figure 2. Limited cross-reactivity of the various mAb for the T cell stimulatory epitopes.

Cross reactivity of the mAb was determined in a direct binding experiment. BSA-coupled peptides encoding the T cell stimulatory epitopes were coated on an Elisa plate and binding of the mAb was determined. Binding of (A) anti-Glia-α9; (B) anti-Glia-γ1; (C) anti-Glia-α20; (D) anti-LMW-glt; (E) anti-HMW-glt mAb to the indicated T cell epitopes. Glia=gliadin, Glt=glutenin, HMW=high molecular weight glutenin.

Monoclonal antibodies and gluten specific T cells recognize an overlapping set of natural gluten derived homologues of the T cell stimulatory epitopes

In previous studies it was shown that human gluten-specific T cells can react to several homologous gluten peptides if these share a certain degree of sequence similarity (16). In order to compare the specificity of the human T cells with that of the gluten specific mAb, natural variants of the Glia-α9, Glia-α20, Glia-γ1, LMW- and HMW-glutenin epitopes were identified by database searches. Subsequently, the corresponding peptides were synthesized and the reactivity of T cells and mAb to these peptides was determined.

Eleven variants of the Glia-α9 T cell epitope (QLQPFPQPQLPYPQP, the minimal 9 amino acid T cell epitope is underlined) (17) were identified that contain single or multiple substitutions. In general, substitutions in the region flanking the minimal epitope affected T cell and mAb recognition in the same way (Figure 3A). Substitutions within the 9 amino acid core sequence at positions 3, 4 and 5 significantly reduced or even abolished both T cell and mAb recognition while a substitution at position 8 affected T cell recognition only. This latter is due to the fact that position 8 is outside the antibody epitope (Table 2).

In total four out of the twelve peptides tested were detected by both T cells and mAb, three were detected by T cells only, two by mAb only and the remaining three by neither (Figure 3A, B). Thus, the specificity of the T cells and the antibody partially overlaps.

A similar analysis was performed for the other antibodies, a graphical representation of the results obtained is shown in Figure 3B. Of 21 variants of the Glia-α20 T cell epitope, six were recognized by T cells and mAb, ten by T cells only and one by mAb only (Figure 3B). Also for the eight variants of the LMW-glutenin epitope and the 33 homologues of the HMW-glutenin epitope it was observed that a subset was detected by both T cells and mAb (Figure 3B). In contrast, of the six variants of the Glia-γ1 epitope only one is recognized by the T cells while two others are recognized by mAb only. Thus, with the exception of the Glia-γ1 mAb, all other mAb react with multiple variants of the T cell epitopes.

Gluten specific mAb recognize their epitopes in pepsin/ trypsin digests of wheat flour

Next, we determined the reactivity of the mAb against a pepsin/trypsin digest of wheat flour. For this purpose the mAb were covalently coupled to sepharose beads and incubated with a pepsin/trypsin digest of gluten. Subsequently, unbound material was removed by extensive washing and the bound material eluted with acid and analyzed by mass spectrometry. This approach was successful with the Glia-α9, Glia-α20, LMW-glutenin and HMW-glutenin mAbs. In all cases the mass spectrometric analysis identified peptides that contained the minimal amino acid sequence for which these mAb are specific. The five most abundant peptides identified for each mAb are shown in Table 3. These mAb thus react with gluten peptides that are naturally formed during digestion in the gastrointestinal tract due to the activity of pepsin and trypsin.

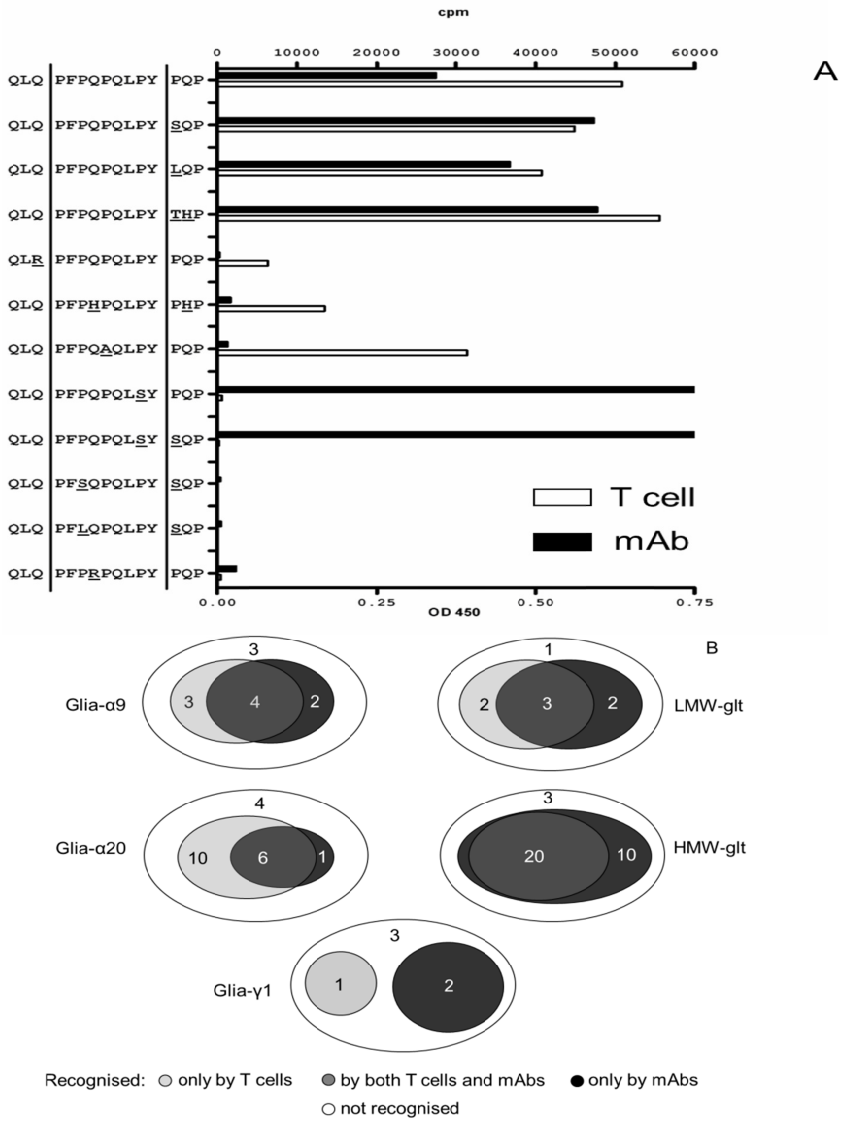

Figure 3. Recognition of naturally occurring gluten variants by T cells and gluten specific mAb.

Database searches identified naturally occurring variants of known T cell stimulatory gluten peptides. The variants were synthesized and tested for recognition by gluten specific T cell clones, in T cell proliferation assay, and mAbs, in a peptide recognition assay. (A). Recognition by T cells and mAb of natural variants of the Glia-α9 T cell epitope (differences with the original epitope are underlined). (B) Overview of the results obtained with the variants of the Glia-α9, Glia-α20, Glia-γ1, LMW-glt and HMW-glt epitopes. The recognition of the LMW-glt epitope was determined using the anti-LMW-1 mAb. With Arabic digits, the number of gluten variants belonging to the category is indicated. mAb=monoclonal antibody, Glia=gliadin, LMW=low molecular weight glutenin, HMW=high molecular weight glutenin.

Table 3. Protein fragments precipitated by gluten specific mAb

Antibody	peptides precipitated	Antibody	peptides precipitated
Anti-Glia-α9[1]	QVQWPQQ**QPFPQPQ**QPF	Anti-Glia-α20	**RPQQPYP**QPQPQ
	PSGQVQWPQQQ**QPFPQPQ**QPF		**RPQQPYP**QPQPQY
	VQWPQQ**QPFPQPQ**QPF		**RPQQPYP**QSQPQY
	PSGQVQWPQQ**QPFPQPQ**QPF		SQPQPF**RPQQPYP**QPQPQ
	PQQ**QPFPQPQ**QPF		SQPQPF**RPQQPYP**QPQPQY
Anti-HMW-Glt[2]	PISPQQPGQGQQSG*QGQPGYYP*TSL	Anti-LMW-glt (1)[3]	SQQQPPFWQQQ**PPFSQQ**QPIL
	PISPQQPGQGQQSG*QGQPGYYP*TS		PQQ**PPFSQQ**QQQPIL
	PISPQQPGQGQQSG*QGQPGYYP*T		PQQ**PPFSQQ**QQPVL
	QPG**QGQQQGYYP**TSPQ		PILPQQ**PPFSQQ**QQPQF
	YPISPQQPGQGQQSG*QGQPGYYP*TS		SQQQ**PPFSQQ**QPIL

Amino acid sequence of gluten peptides detected by the gluten-specific mAb in a pepsin/trypsin digest of gluten. The five most abundant peptides are shown. Mab epitopes are in bold, variants of the epitopes are in italic.
[1] Anti-Glia-α9=anti gliadin alpha-9 antibody
[2] Anti-HMW-Glt=anti high molecular weight glutenin antibody
[3] Anti-LMW-Glt=anti low molecular weight glutenin antibody

Gluten specific antibodies show the presence of T cell stimulatory epitopes in various gluten proteins

To study the suitability of the mAb for the detection of gluten proteins, these were extracted with isopropanol/DTT from a wheat variety and separated on SDS-PAGE. Subsequently, the proteins were either stained directly with Coomassie blue or, after transfer to a membrane, by the gluten specific mAb in a Western blot analysis (Figure 4). To identify the proteins stained by the various mAb in the Western blot analysis, gel slices were excised from the Coomassie blue stained gel at positions corresponding to the mAb stained bands. Subsequently the proteins in these gel slices were isolated, digested with chymotrypsin and analyzed by mass spectrometry. All chymotrypsin fragments isolated from the gel slice corresponding to the proteins stained by the HMW-specific mAb (Figure 4, lane 2, proteins 1 and 2) were found to be derived from HMW-glutenin proteins. These fragments contained multiple copies of the minimal amino acid sequences recognized by both the mAb and T cells specific for HMW-glutenin (results not shown). Similarly, both of the proteins recognized by the anti-LMW-1 mAb (Figure 4, lane 3, proteins 3 and 4) were identified as LMW-glutenin proteins and contained the minimal amino acid sequences recognized by the mAb and the

T cell (results not shown). The gel slices corresponding to the regions stained by the Glia-α9, Glia-α20 and Glia-γ1 specific mAb (Figure 4, lane 4, 5 and 6 proteins 5-8) were found to contain α/β-gliadin proteins that contained both the Glia-α9 and Glia-α20 T cell and mAb epitopes (Figure 4 lanes 4, 5 and 6, proteins 5, 6, 7 and 8). In addition, a γ-gliadin protein (Figure 4, lanes 4, 5, 6 protein 8) and a LMW-glutenin protein that contained the LMW-1 mAb epitope (1) (Figure 4, lane 4, 5 and 6, protein 5, 6, 7 and 8) were identified.
Thus, the mAb are useful for the detection of gluten proteins that harbour harmful peptides involved in CD.

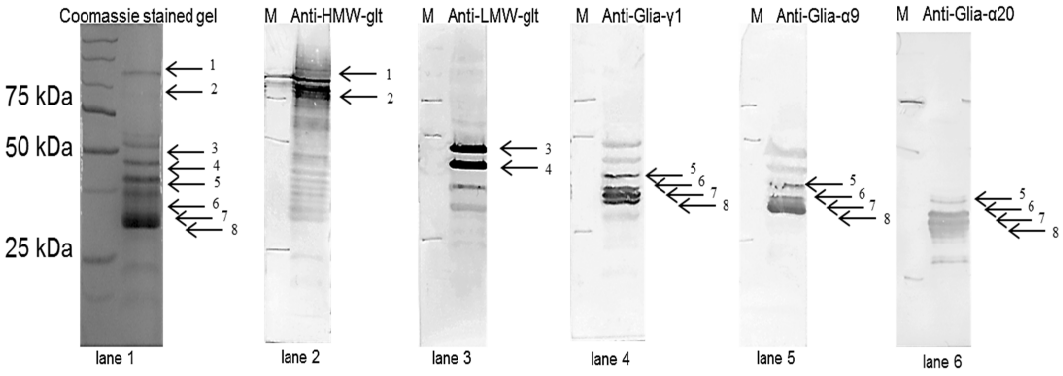

Figure 4. Detection of gluten proteins by Western blot.

Gluten proteins were extracted from flour of a wheat variety and separated by SDS PAGE. The proteins were either stained directly (lane 1) or after blotting to a PDVF membrane and visualised with the mAb specific for HMW- glt (lane 2), LMW- glt 1 (lane 3), Glia-γ1 (lane 4), Glia-α9 (lane 5) and Glia-α20 (lane 6). Proteins recognised by the antibodies (numbered and indicated by arrows) were excised from the directly stained gel and digested with chymotrypsin. After extraction the identification of the isolated proteins was determined by a combination of tandem MS/MS mass spectrometry and bioinformatics. M is the mass marker. Glia=gliadin, LMW-glt-low molecular weight glutenin, HMW-glt=high molecular weight glutenin.

Gluten specific antibodies show the presence of gluten homologs in barley, rye triticale and oat

Previously, we have described that in the storage proteins of barley, rye and oat sequences are present that are highly similar to the T cell stimulatory epitopes in gluten (Table 4) and that some of them are recognized by gluten-specific T cells (14). We have now determined if the gluten-specific mAb likewise react with the homologous peptides from barley, rye and oat. The Glia-α9 and Glia-α20 mAbs were found to primarily react with secalin and hordein derived peptides but not with those from avenins (Fig. 5A and 5B). In contrast, the Glia-γ1 mAb strongly reacted with avenin peptides and not or hardly with the hordein and secalin peptides (Figure 5C).

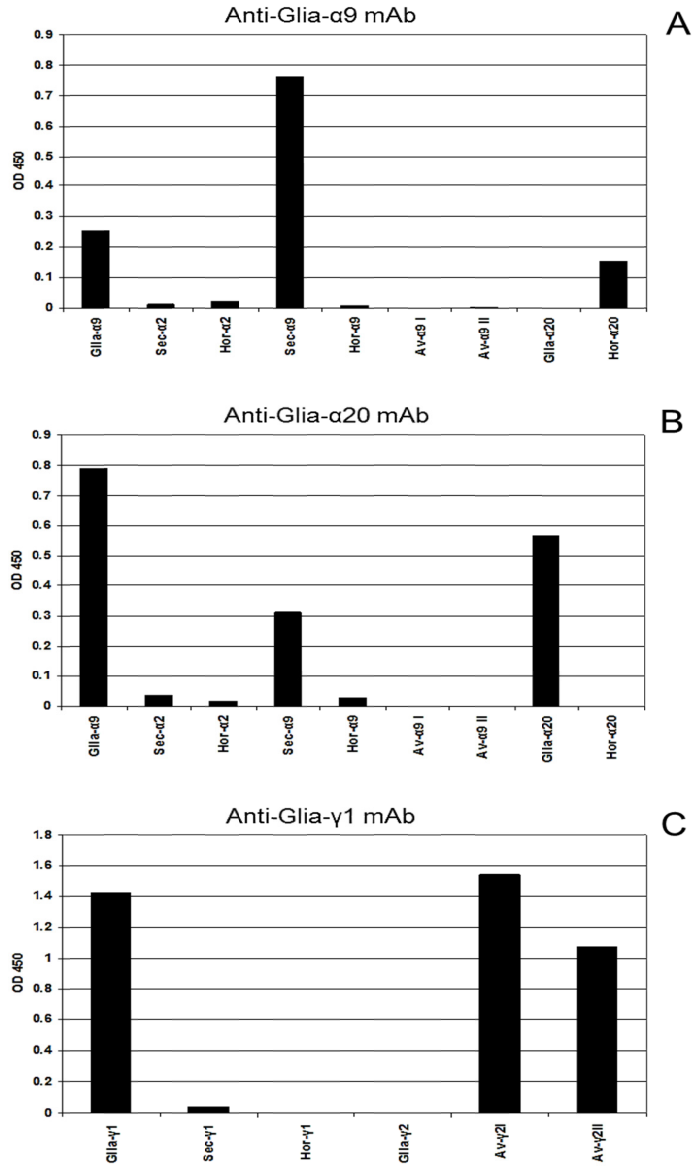

Figure 5. Recognition of hordein, secalin and avenin peptides by gliadin specific mAb.

Gluten peptides and homologous peptide sequences in hordeins, secalins and avenins identified by database screening (14), were tested for recognition in a direct binding assay, by the gluten specific mAb. (A) anti-Glia-α9 mAb, (B) anti-Glia-α20 mAb, (C) anti-Glia-γ1 mAb. Glia=gliadin, Sec=secalin, Hor=hordein, Av=avenin.

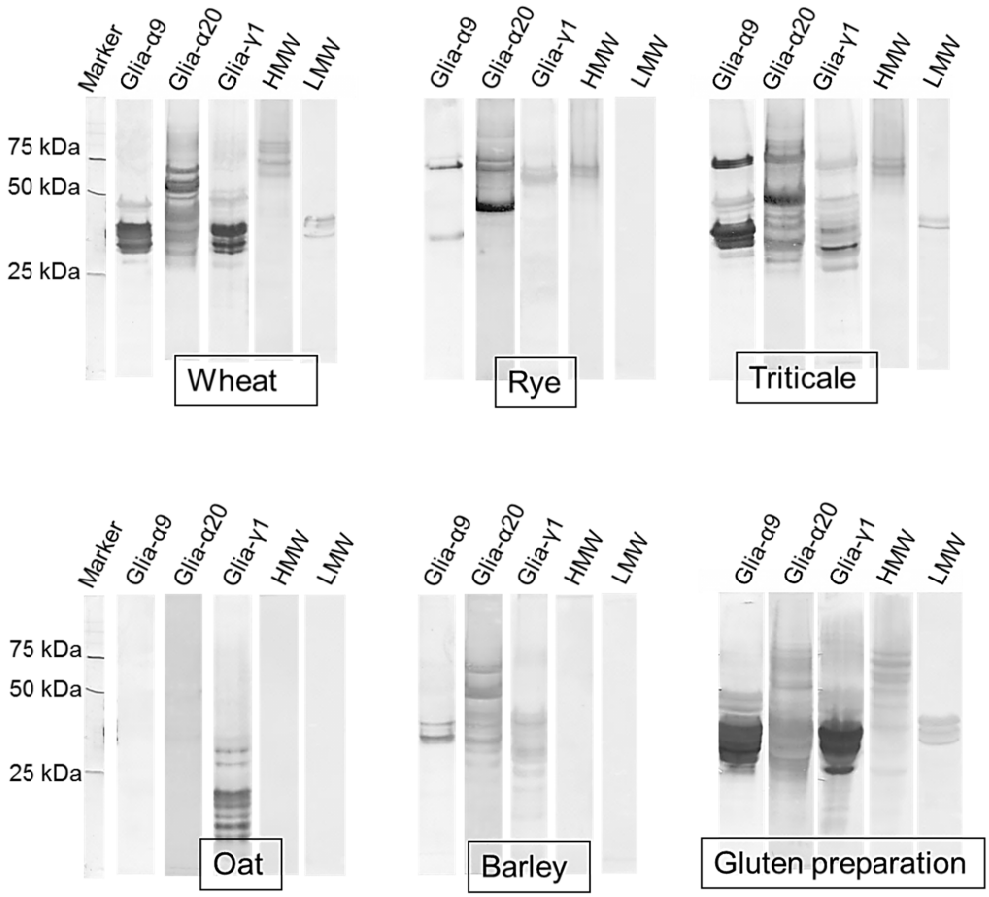

Figure 6. Detection of gluten homologues present in barley, rye, oats and triticale by Western blot analysis.

Protein extracts were prepared from wheat, rye, triticale, oats and barley. A commercial gluten preparation was used as a positive control. After separation by SDS-PAGE, the protein extracts were transferred to a PVDF membrane and analyzed by Western blot. The presence of gluten epitopes were detected using the anti-Glia-α9, anti-Glia-α20, anti-Glia-γ1, anti-LMW glutenin and anti-HMW glutenin antibodies as indicated. Glia=gliadin, LMW-glt-low molecular weight glutenin, HMW-glt=high molecular weight glutenin.

Table 4. Gluten epitopes and the homologous peptide sequences found in hordein, secalin and avenin.

Designation	Sequence
Gliadin α2/ α9	PYLQL**QPFPQPQ**LPYPQPQLPYPQPQ
Secalin α2	QPFPQPQQPFPQSQ
Hordein α2	QQFPQPQQPFPQQP
Gliadin α2/α9	PYLQL**QPFPQPQ**LPYPQPQLPYPQPQ
Secalin α9	PQQPFPQPQQPFPQ
Hordein α9	PQQPFPQPQQPFRQ
Avenin α9I	QYQPYPEQQEPFVQ
Avenin α9II	QYQPYPEQQQPFVQ
Gliadin α20	PQP**FRPQQPY**PQQP
Hordein α20	QQPFPPQQPFPQQP
Gliadin γ1	PQPQQPQQSFPQ**QQRPFI**QPSLQ
Secalin γ1	PIPQQPQQSFPQQPQRPEQQFPQ
Hordein γ1	QQPFPPQQAFPQQPPFWPQQPQQ
Gliadin γ2	QQPFPQQPQQPFPQ
Avenin γ2I	QQPFVQQQQQPFVQ
Avenin γ2II	QQPFVQQQQPFVQQ

Cereal storage protein sequences were obtained by database searches using the protein family as keyword (15) (Table 1). T cell stimulatory sequences are underlined. Sequences recognized by mAb are shown in bold. The elongated residues are indicated in grey.

Table 5. Recognition of various cereals by gluten specific mAb.

Sample	mAb				
	Glia-α9[1]	Glia-α20	Glia-γ1	HMW[2]	LMW[3]
Wheat	+	+	+	+	+
Rye	+	+	+	+	-
Triticale	+	+	+	+	+
Oat	-	-	+	-	-
Barley	+	+	+	-	-
Gluten preparation	+	+	+	+	+

Reactivity of the mAb against gluten and gluten-like proteins in wheat, barley, rye, triticale and oat. Gluten preparation was used as control.
[1] Glia=gliadin
[2] HMW=high molecular weight
[3] LMW=low molecular weight

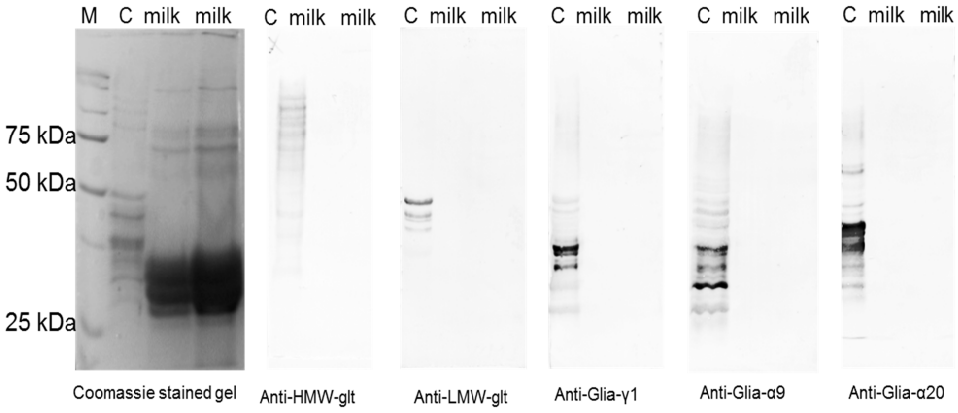

Figure 7. No cross reactivity of gluten specific antibodies with milk proteins.

2 different amounts of a milk water solution were separated by SDS-PAGE. The proteins were either stained directly with Coomassie or after transfer to a PDVF membrane with the mAb specific for HMW- glt, LMW- glt 1, Glia-γ1, Glia-α9 and Glia-α20. M is mass marker, C is the gluten preparation used as positive control, milk represents the milk sample.

To demonstrate that the mAb were capable of detecting hordeins, secalins and avenins, protein extracts of five cereals (wheat, barley, oat, rye and triticale) were prepared and separated by SDS-PAGE. Subsequently, the proteins were stained with the mAb in a Western blot analysis (Figure 6, Table 5). A commercial gluten preparation was used as a positive control. As expected the staining pattern of the gluten preparation and the wheat protein extract were very similar. In contrast, distinct staining patterns were observed for oat, rye and barley, while the staining pattern of triticale, a hybrid containing both the wheat and the rye genome, closely matches the sum of the staining patterns of wheat and rye. The results (Figure 6, Table 5) indicate that the Glia-α9 mAb reacts with all cereals except oat. The HMW mAb only reacts with wheat and rye and the LMW mAb only with wheat. The reactivity of the HMW mAb with rye is presumably based on the presence of proteins in rye that have a high degree of homology with both the x- and y-type of HMW-glutenins of wheat (18). The staining patterns obtained with the Glia-α20 and Glia-γ1 mAb are more complex, the latter having a high affinity for the oat proteins.

The results also indicate that the mAb-staining patterns obtained are distinct for each cereal. This may be useful for the identification of the type of (contaminating) cereals present in (gluten-free) food products.

Gluten specific antibodies do not react with milk proteins

As the gluten specific antibodies are used in assays where milk is employed as a blocking agent we tested if the antibodies cross-react with proteins in milk as an additional control for the assay. For this, a Western blot analysis was performed. Milk was diluted in water and the proteins precipitated with acetone. Subsequently the proteins were separated on SDS-PAGE, transferred to blots and stained with the antibodies against Glia-α9, Glia-α20, Glia-γ1, LMW- and HMW-glutenin gluten epitopes. None of the antibodies reacted with proteins in milk while gluten proteins were clearly detected (Figure 7).

Comparison of Ridascreen® Gliadin kit and the Home made Elisa for the Glia-α9 epitope.

A commercial test kit is now available based on the R5 monoclonal antibody specific for the sequence QQPFP. As this sequence partially overlaps with that recognized by the mAb specific for the alpha-9 T cell epitope (QPFPQPQ) we have compared the reactivity of the alpha-9 specific mAb with that of the R5 antibody. For this purpose we tested a number of samples with both our mAb against the alpha-9 peptide and with the commercially available R5 based test kit (Table 6). Gluten-free samples tested were negative in both assays (not shown). Gluten containing samples could easily be detected by both assays although the actual values obtained varied. In general the values obtained with the competition assay were higher as those obtained with the R5. This may be due to the standards used in the two assays, a complex gliadin preparation in the case of the R5 based assay versus synthetic peptides for the competition assay. An in depth comparison of these two assays is planned.

Table 6. Comparison of Ridascreen® Gliadin kit and the Glia-α9 specific assay.

Sample	Ridascreen® gliadin [ppm][1]	Glia-α9[2] specific assay [ppm]
toast "LePoole"	1.52	8.08
butter biscuits "LePoole"	20.67	27.19
cake bakery "De Wijn"	3.15	18.66
flour "LePoole Twelloo's" quinoa	2.81	11.23
couscous "Nestle"	>>	349.9

Gluten containing samples were tested with both the Ridascreen® gliadin, based on the R5 antibody, and the assay specific for the Glia-α9 epitope developed in our laboratory.
[1] ppm=part per million
[2] Glia=gliadin

DISCUSSION

It is well established that celiac disease is caused by intolerance to gluten. With few exceptions celiac disease only develops in individuals that are either HLA-DQ2 or HLA-DQ8 positive. The extraordinary strong association between these HLA-DQ molecules and the development of celiac disease points to a crucial role for these HLA-DQ molecules in disease development. Recent work has revealed the basis for this association: HLA-DQ2 and HLA-DQ8 can bind gluten-derived peptides and present these to inflammatory T cells that are present in the small intestine of celiac disease patients, leading to disease (19-21) Consequently, the gluten-derived peptides that can bind to HLA-DQ2 or HLA-DQ8 and stimulate T cells are the true culprits and should not be present in food intended for consumption by celiac disease patients.

Importantly, such T cell stimulatory gluten peptides have now been identified in α- and γ-gliadin and LMW- and HMW-glutenin. Both gliadins and glutenins are multiprotein families with a high degree of sequence homology between different members. Therefore, variants of the identified T cell stimulatory peptides are known that can also elicit T cell responses (7). Moreover, peptides with similar T cell stimulatory properties have been identified in barley, rye and oat as well (15). In order to guarantee the safety of food products intended for the gluten-free market it would therefore be highly desirable to use tools that detect as many of the potentially harmful gluten and gluten-like fragments as possible.

With this in mind, mAbs were raised against non-deamidated forms of known T cell stimulatory peptides present in gluten. This will allow detection of native gluten peptides that upon deamidation by tissue transglutaminase in the small intestine can trigger an immune response. We aimed at detecting both the alpha- and gamma-gliadins and HMW and LMW glutenins. With these antibodies we can thus monitor the presence of 4 different classes of gluten proteins which is a large improvement compared to the existing assays based on the R5 mAb that only recognizes gliadin proteins.

In this paper we provide a detailed analysis of the reactivity of these antibodies against gluten proteins, gluten peptides, and gluten-like proteins present in barley, rye and oat. The minimal amino acid sequences detected by these mAb were determined and found to partly overlap with that of T cell stimulatory gluten sequences. Moreover, as gluten-specific T cells do (13), the mAb were found to react not only with the peptide against which they were raised but also with homologous gluten proteins and peptides present in wheat, barley, rye and oat. In addition the proteins recognised by the mAb did contain epitopes involved in CD. By Western blot analysis these antibodies were found to give characteristic staining patterns depending of the cereal being analyzed, a property that may be useful for the identification of the type of cereal present in and/or contaminating particular food products.

Next to its reactivity with gluten and gluten-like proteins and peptides, the mAb against the Glia-γ1 epitope also reacted strongly with oat, a finding that may be explained by the homology between the N-terminal sequence of the Glia-γ1 T cell epitope: QQRPFI and an amino acid sequence that is frequently present in the α- and γ-avenins: QQQPFV. Similarly, oat protein can be recognized by Glia-γ1 specific T cells (13), although it should be indicated that the specificity of the mAb and T cells overlap only minimally (Table 2).

In separate studies it was tested if the antibodies react with proteins from teff, rice and maize, cereals that are non-toxic for celiac disease patients. In all instances we observed reactivity with wheat, rye and/or barley only (22).

The Codex Alimentarius defines gluten-free foods as those whose gluten contents is below 200 ppm for wheat starch containing food products and below 20 ppm for naturally gluten-free food products. In a new draft for the standards used for gluten-free food products the level of 100 ppm for food rendered gluten-free is recommended (//ftp.fao.org/codex/alinorm07/al30_26e.pdf). The best known commercially available assays for the detection of gluten are sandwich ELISA's based on the R5 antibody, which is specific for a common sequence present in α-, -β, and -γ gliadins (23), and on an antibody directed against a sequence in ω-gliadin (24). However, as these sequences do not match T cell stimulatory gluten peptides these assays do not measure the presence of harmful fragments for CD patients. Moreover, these assays fail to detect the HMW- and LMW-glutenins while there is mounting evidence that these proteins are harmful for CD patients as well (7;8;17;25). With the availability of antibodies specific for α- and γ- gliadins as well as LMW- and HMW-glutenins, assays can now be developed that overcome these problems. Moreover, as these antibodies do not only react with gluten proteins but with peptides as well, they are suitable for use in competition assays in which not only intact gluten proteins can be measured but also gluten peptides of sizes recognised by T cells. This is of particular interest as gluten hydrolysates are often used in the food industry and these cannot be measured in sandwich ELISA's.

In conclusion, the antibodies described in this study allow a comprehensive screen for the presence of harmful gluten and gluten-like peptides and proteins in foods intended for consumption by CD patients with a level of detail that is as yet unprecedented. This will likely contribute to food safety and thereby the quality of life of CD patients.

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The authors contribution was as follows: CM performed experiments with the monoclonal antibodies, YKW tested the hybridoma producing antibodies, PvV and AdR performed the mass spectrometry measurements and analyzed the results, JWD was involved in the synthesis of the synthetic peptides, LD obtained the monoclonal antibodies, tested them and developed monoclonal antibody based assays. CM, FK and LD designed the experiments, analyzed the results and wrote the manuscript.

*None of the author**s** has personal or financial conflicts of interests.*

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Chapter 3

A universal approach to eliminate antigenic properties of alpha-gliadin peptides in celiac disease

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ABSTRACT

Celiac disease is caused by an uncontrolled immune response to gluten, a heterogeneous mixture of wheat storage proteins, including the α-gliadins. It has been shown that α-gliadins harbor several major epitopes involved in the disease pathogenesis. A major step towards elimination of gluten toxicity for celiac disease patients would thus be the elimination of such epitopes from α-gliadins. We have analyzed over 3000 expressed α-gliadin sequences from 11 bread wheat cultivars to determine whether they encode for peptides potentially involved in celiac disease. All identified epitope variants were synthesized as peptides and tested for binding to the disease-associated HLA-DQ2 and HLA-DQ8 molecules and for recognition by patient-derived α-gliadin specific T cell clones. Several specific naturally occurring amino acid substitutions were identified for each of the α-gliadin derived peptides involved in celiac disease that eliminate the antigenic properties of the epitope variants. Finally, we provide proof of principle at the peptide level that through the systematic introduction of such naturally occurring variations α-gliadins genes can be generated that no longer encode antigenic peptides. This forms a crucial step in the development of strategies to modify gluten genes in wheat so that it becomes safe for celiac disease patients. It also provides the information to design and introduce safe gluten genes in other cereals, which would exhibit improved quality while remaining safe for consumption by celiac disease patients.

INTRODUCTION

Celiac Disease (CD) is an intestinal T cell-mediated disease caused by the gluten fraction of wheat or the homologous proteins from barley or rye. CD has prevalence between 0.5 and 2% in human populations [1] and is characterized by a chronic intestinal inflammation upon ingestion of gluten proteins. Recently, the molecular aspects have been comprehensively addressed in several review papers [2-5]. In short, in CD patients CD4+ T cells are present in the lamina propria that secrete interferon-gamma upon recognition of gluten-derived peptides bound to HLA-DQ2 or HLA-DQ8 molecules present on antigen presenting cells. Strikingly, most of the gluten peptides implicated in CD require modification by the enzyme tissue transglutaminase before they can bind to the disease-predisposing HLA-DQ molecules and trigger T cell responses [2-5]. In addition to the adaptive CD4+ T cell response to gluten, CD is characterized by the upregulation of IL-15, an intraepithelial T cell infiltrate expressing the NKG2D receptor, and the overexpression of a ligand for NKG2D (MICA) [6, 7].

Many gluten peptides with T cell stimulatory properties have now been identified. Such peptides have been found in wheat α-, γ-, and ω-gliadins as well as in low molecular weight (LMW) and high molecular weight (HMW) glutenins [8-14]. Several studies have demonstrated that peptides derived from α-gliadins induce strong T cell responses in the large majority of patients, while responses to the other peptides are less frequently found [8-10, 13]. An α-gliadin derived 33-mer peptide (amino acid sequence LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) was identified that encodes six partially overlapping T cell epitopes and has very potent T cell stimulatory properties [13]. It harbours the p56-75 peptide (LQLQPFPQPQLPYPQPQLPY) that has been identified as the dominant gluten epitope [9, 10]. Furthermore, α-gliadins are the only gluten molecules that harbor the p31-43/49 peptide that has been implicated in the innate immune response induced by gluten [7].

The α-gliadins are a gene family encoded by the *Gli-2* loci, *Gli-A2, Gli-B2 and Gli-D2,* located on the short arm of three homoeologous chromosomes (6AS, 6BS and 6DS) of hexaploid bread wheat (*Triticum aestivum L.*). These loci may contain from 25-35 to even 150 α-gliadin genes per haploid genome [15-17], although most of these (72-95%) are presumably pseudogenes [16,17]. Sequencing of genomic α-gliadin clones from hexaploid bread wheat enabled to differentiate the sequences according to their loci *Gli-A2, Gli-B2* and *Gli-D2* based on genome-specific SNPs [16,17]. Relevant for CD, the occurrence and frequency of the HLA-DQ2 epitopes DQ2-Glia-α1, DQ2-Glia-α2 and DQ2-Glia-α3 (previously designated Glia-α20, ref. 12] and the HLA-DQ8 T-cell epitope DQ8-Glia-α1 also differs between the loci [17]. This was corroborated by the observation that T cell clones specific for the DQ2-Glia-α2 epitope did not recognise gluten derived from diploid species carrying the S-genome, ancestrally related to the B genome of bread wheat, while gluten derived from diploid A- and D-genome species was recognized [18]. Variation in T cell stimulatory capacity of cereal-derived gluten was observed with other T cell clones as well [19-21]. Indeed, differences have been ob-

served in the T cell stimulatory capacity of pasta and bread wheat varieties [22,23], but none were safe for CD patients.

Given the overall importance of the α-gliadins in CD we set out to determine the naturally existing sequence variation in CD epitopes as deduced from α-gliadin transcripts from developing wheat grains. The immunogenic potential of these epitope variants was subsequently tested in T-cell proliferation assays. This produced insight in which key amino acid changes are sufficient to abolish T-cell recognition. Finally, we verified that the observed differences in antigenicity of α-gliadin peptides derived from diploid species corresponded to differences in the antigenicity of the gluten from these species, ancestrally related to bread wheat. Altogether the results offer a molecular basis for differential CD toxicity of the wheat genomes. Based upon these results we present a rational strategy to develop genes that encode α-gliadins that are safe for consumption by celiac disease patients.

RESULTS

Genetic variation in α-gliadin (*Gli-2*) transcripts

In total 3022 expressed α-gliadin sequences (expressed sequence tags and mRNA sequences from NCBI and Unigene) originating from 11 different *T. aestivum* L. varieties were analyzed. These α-gliadin transcripts were grouped into 55 contigs with at least 90% sequence homology. Forty per cent of the α-gliadin transcripts clustered with A genomic sequences and were attributed to locus *Gli-A2*, 35% originated from *Gli-D2* and only 25% came from *Gli-B2* (Figure. S1). After tracing all non-synonymous DNA polymorphisms 83 different transcript contigs were obtained for the 3' region of the gene that each contained at least four sequence equivalents. This indicates a high sequence diversity among expressed α-gliadin sequences in these 11 *T. aestivum* L. varieties.

Table 1. Amino acid sequences of α-gliadin derived peptides.

	Immune response	Restriction element	Core-Sequence
DQ2-Glia-α1	Adaptive	HLA-DQ2	P{F/Y}PQPQLPY
DQ2-Glia-α2	Adaptive	HLA-DQ2	PQPQLPYPQ
DQ2-Glia-α3	Adaptive	HLA-DQ2	FRPQQPYPQ
DQ8-Glia-α1	Adaptive	HLA-DQ8	QGSFQPSQQ
P31-43	Innate	not applicable	PGQQQPFPPQQPY

Five antigenic peptides derived from α-gliadins are known to be involved in celiac decease. For the peptides that can provoke an adaptive immune response in CD patients (HLA-DQ2 restricted or HLA-DQ8 restricted) the minimal 9-mer "canonical" epitope cores are shown. One peptide, ***p31-43,*** *is known to be involved in an innate immune response observed in CD. For each of the epitopes is specified the name, which immune response it evokes, the restriction element and the amino acid sequence.*

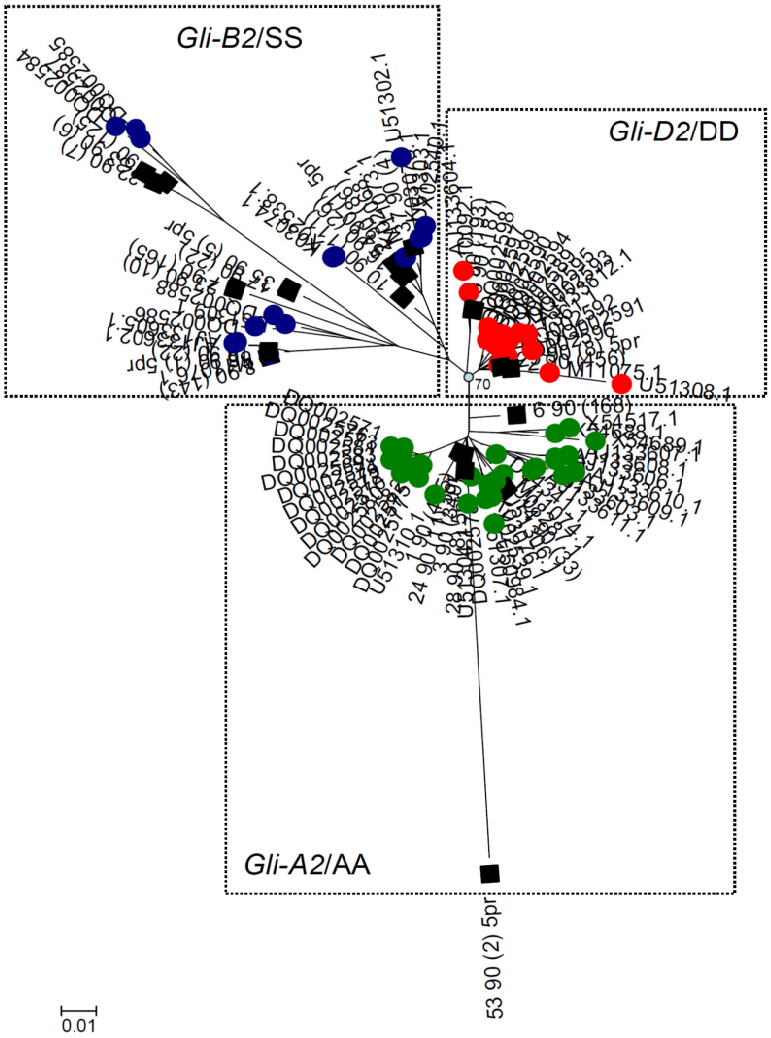

Figure S1. Phylogenetic analysis of α-gliadin sequences.

A neighbor-joining tree was made with 55 EST consensus nucleotide sequences from hexaploid bread wheat together with 56 genomic DNA sequences derived from the diploid wheat species T. monococcum (A genome, green dots), T. speltoides (S/B genome, blue dots) and Aegilops tauschii (D genome, red dots), after alignment using Clustal W. The EST sequences (black dots) can be assigned to their locus in hexaploid bread wheat as they cluster into the same three groups as the sequences from the diploid species (A genome = locus Gli-A2, S/B genome = locus Gli-B2, D genome = locus Gli-D2 [17]).

Variants of T cell stimulatory and innate stimulatory sequences

The N-terminal region of α-gliadins contains the p31-43 epitope implicated in the innate immune response, and the immunodominant DQ2-Glia-α1 and DQ2-Glia-α2 epitopes as well as the DQ2-Glia-α3 T cell epitope. The carboxyl-terminal part encodes the immunodominant DQ8-Glia-α1 T cell epitope (Table 1). To obtain information on the immunogenic potential of the various α-gliadins, the translated amino acid sequences for each locus were checked for the presence of canonical T cell epitopes and variants thereof. Table 2-5 present the most frequently expressed epitope variants (>5 transcripts each).

Table 2. HLA-DQ2-Glia-α1 epitope variants expressed in bread wheat.

	Gli-A2	*Gli-B2*	*Gli-D2*	N
PFPQPQLPY	466		520	986
P**Y**PQPQLPY			93	93
PF**L**QPQLPY	106			106
PF**S**QPQLPY	94			94
PFPQPQL**S**Y	7		54	61
PFP**H**PQLPY			29	29
PFPQ**A**QLPY			6	6
Total	673		702	1375

Expressed variants of the DQ2-Glia-α1 epitope represented by ≥5 transcripts and the number of transcripts per epitope variants per chromosomal locus (Gli-A2, Gli-B2 or Gli-D2). N= total transcript count per variant. In bold: amino acid variation.

Table 3. DQ2-Glia-α2 epitope variants expressed in bread wheat.

	Gli-A2	*Gli-B2*	*Gli-D2*	N
PQPQLPYPQ			607	607
PQPQLPY**S**Q	431			431
FPPQLPYPQ		382		382
LQPQLPY**S**Q	106			106
FLPQLPYPQ		31		31
SQPQLPY**S**Q	85			85
PQPQL**S**YPQ			54	54
PQP**H**LPYPQ			30	30
P**H**PQLPYPQ			29	29
PQPQL**S**Y**S**Q	7			7
PQ**A**QLPY**S**Q			6	6
PQPQ**PQ**YPQ		6		6
Total	629	419	726	1774

Expressed variants of the DQ2-Glia-α2 epitope represented by ≥5 transcripts and the number of transcripts per epitope variants per chromosomal locus (Gli-A2, Gli-B2 or Gli-D2). N= total transcript count per variant. In bold: amino acid variation.

We observed that canonical DQ2-Glia-α1 (Table 2) and DQ2-Glia-α3 epitopes (Table 4) were only present in *Gli-A2* and *Gli-D2* transcripts and that the canonical DQ2-Glia-α2 motif (Table 3) was unique for *Gli-D2* transcripts (72% of all *Gli-D2* transcripts contained at least one DQ2-Glia-α2 epitope core). DQ8-Glia-α1 canonical epitopes were present in *Gli-D2* and *Gli-B2* transcripts only (Table 5). The canonical p31-43 motif was not restricted to transcripts from a specific locus and was found in transcripts from all α-gliadin loci.

Table 4. DQ2-Glia-α3 epitope variants expressed in bread wheat

	Gli-A2	*Gli-B2*	*Gli-D2*	N
FRPQQPYPQ	650		449	1099
*F**P**PQQPYPQ*	732	687	606	2025
*F**P**S**QQPYPQ*		179	8	187
FRPQQ**S**YPQ			157	157
F**P**PQQPYP**H**		148		148
F**PA**QQPYPQ		56		56
F**P**PQQ**S**YPQ		18		18
F**PG**QQPYPQ		20		20
F**Q**PQQPYPQ	13			13
F**L**PQQPYPQ			8	8
FRPQQ**Q**YPQ			6	6
Total	1395	1108	1234	3737

Expressed variants of the DQ2-Glia-α3 epitope represented by ≥5 transcripts and the number of transcripts per epitope variants per chromosomal locus (Gli-A2, Gli-B2 or Gli-D2). In italics: DQ2-Glia-α3 variants located on the position of the innate responsive element, p31-43. N= total transcript count per variant. In bold: amino acid variation.

Table 5. DQ8-Glia-α1 epitope variants expressed in bread wheat.

	Gli-A2	*Gli-B2*	*Gli-D2*	N
QGSFQPSQQN		186	381	567
QGSF**R**PSQQN	409			409
QG**F**FQPSQQN			114	114
QGSF**R**P**F**QQN	103			103
Q**V**SFQPSQ**L**N		112		112
QGSFQ**S**SQQN		111		111
QGSFQP**F**QQN		4	27	31
QG**F**FQP**F**QQN			6	6
Total	512	413	528	1453

Expressed variants of the DQ8-Glia-α1 epitope, represented by ≥5 transcripts and the number of transcripts per epitope variants per chromosomal locus (Gli-A2, Gli-B2 or Gli-D2). N= total transcript count per variant. In bold: amino acid variation

In addition to the canonical epitope motifs, a large series of sequence variants with one or two amino acid substitutions were detected (Table 2-5). The large majority of the *Gli-B2* gliadins contained sequence variants of the DQ2-Glia-α1, DQ2-Glia-α2, and DQ2-Glia-α3 epitopes with two amino acid substitutions. Furthermore, *Gli-A2* transcripts harbored a proline to serine substitution at position 8 (p8) of the DQ2-Glia-α2 epitope and a sequence variant (QGSF**R**P(S/F)QQN, amino acid substitution in bold) of the DQ8-Glia-α1 epitopes. Importantly, the 33-mer peptide (LQLQPFPQPQLPYPQ-PQLPYPQPQLPYPQPQPF) that is highly resistant to degradation in the gastrointestinal tract and contains six overlapping DQ2-Glia-α1 and DQ2-Glia-α2 epitopes, conferring superior T cell stimulatory properties [13], was only observed in a subset of the α-gliadins from the *Gli-D2* locus and never in the α-gliadins from the *Gli-A2* and *Gli-B2* loci. The latter expressed substantially truncated versions of the 33-mer (Figure S2).

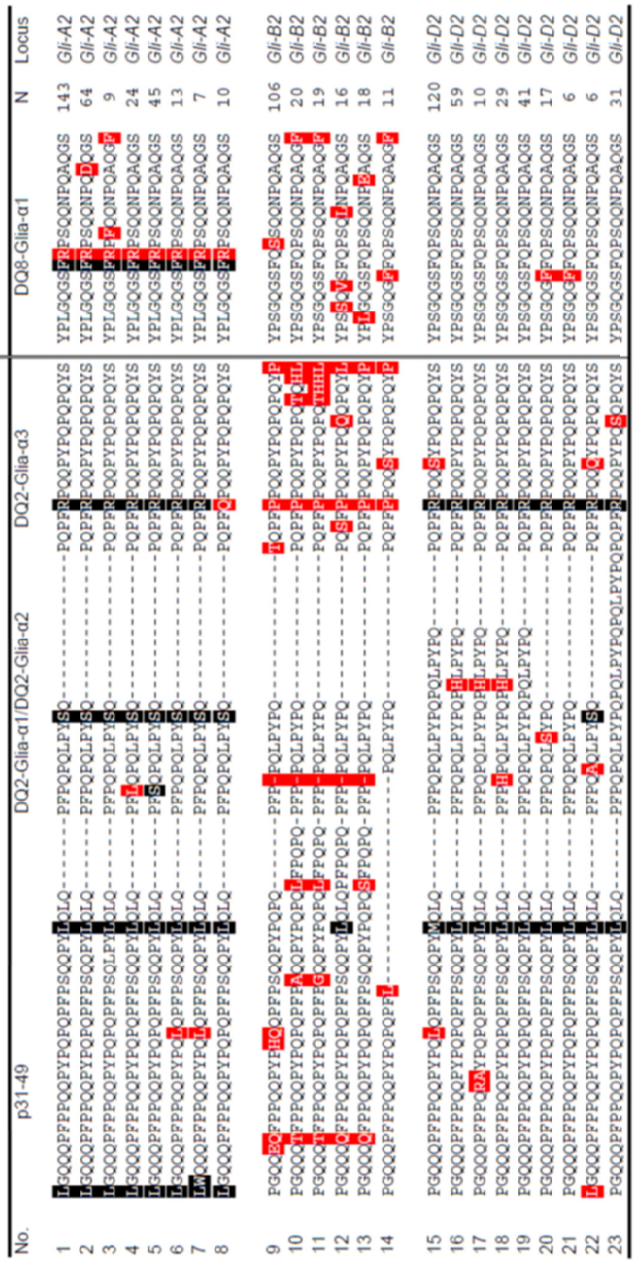

Figure S2. Sequence variation in the N-terminal and C-terminal regions of α-gliadin genes from hexaploid wheat.

The 23 most frequently found expressed sequence tag (EST) contigs were translated, the amino acid sequences were aligned and grouped per chromosomal location (the Gli-A2 locus on chromosome 6AS, Gli-B2 on 6BS, and Gli-D2 on 6DS) to present the variation in various gluten epitope regions. Note the large differences in the number of times (N) each sequence was present in the set of ESTs. In red: amino acid variation in the sequence. In black: chymotrypsin or trypsin sites (>72% affinity).

Table 6. T cell proliferation and HLA-DQ2 binding capacity of DQ2-Glia-α variants.

No	Peptide	locus	IC50 DQ2-Glia-α1	Glia-α1 T cell clone	IC50 DQ2-Glia-α2	Glia-α2 T cell clone
1	QLQPFPQPELPYPQP**E**	*Gli-D2*	5	+	18	+
2	QLQPFPQPELPYPQPQ	*Gli-D2*	5	+	34	+
3	QLQPFPQ**A**ELPY**S**QPQ	*Gli-D2*	8	±	11	-
4	QLQPFPQPEL**S**YPQPQ	*Gli-D2*	12	-	24	-
5	QLQ**R**PFPQPELPYPQP**E**	*Gli-D2*	14	±	19	+
6	QLQPFPQPELPY**TH**	*Gli-D2*	21	+	21	-
7	QLQPFP**H**PELPYPQPQ	*Gli-D2*	42	+	41	±
8	QLQPF**S**QPELPY**S**QPQ	*Gli-A2*	7	±	32	-
9	QLQPFPQPELPY**S**QPQ	*Gli-A2*	57	+	41	-
10	QLQPFPQPELPY**S**QPE	*Gli-A2*	67	+	24	-
11	QPQPF**L**–PELPYPQPE	*Gli-B2*	4	-	4	±
12	QPQPF–QPELPYPQPE	*Gli-B2*	8	-	8	+
13	QPQ**E**FP–PELPYPQPE	*Gli-B2*	45	-	45	-
14	QPQ**Q**FP–PELPYPQPE	*Gli-B2*	86	-	86	-
15	QPQPFP–PELPYPQ**T**QP	*Gli-B2*	nd	-	nd	-

			IC50 DQ2-Glia-α3	Glia-α3 T cell clone
16	QPFRPEQPYPQPQPQ	*Gli-A2/D2*	35	+
7	QPF**P**PEQPYPQPQPQ	*Gli-A2/D2/ B2*	3080	-

			IC50 DQ8-Glia-α1	DQ8-Glia-α1 T cell clone
18	LGEGSFQPSQENP	*Gli-D2/B2*	16	+
19	LGEGSF**R**PSQENP	*Gli-A2*	33	-
20	LGEG**F**FQPSQENP	*Gli-D2*	nd	-

Variants of the DQ2-Glia-α1 and DQ2-Glia-α2 epitopes as encoded by the α-gliadin transcriptome were synthesized as deamidated 14- to 17-mer peptides (column 1, underlined: DQ2-Glia-α1/α2 epitope region) and tested for stimulation of DQ2-Glia-α1 and DQ2-Glia-α2 specific T cell clones in a proliferation assay. ± = 100 times reduced T cell stimulation compared to the 'canonical' epitope; - = 1000 times reduced T cell stimulation compared to the 'canonical' epitope. For each epitope shorter versions of the peptide variant, including the putative epitope core flanked by at least two amino acids, were tested in a cell free in vitro peptide binding assay for binding to HLA-DQ2 antigen presenting cells (lysates from HLA-DR3/DQ2 positive EBV-transfored B-cells). IC50 DQ2-Glia-α1/α2=mean value of the half maximal inhibitory concentration (IC_{50}) returned by the binding assays for respectively DQ2-Glia-α1 and DQ2-Glia-α2 epitope variants. IC_{50} values were calculated based on the observed competition between the tested peptides and biotin-labelled indicator peptides and indicate the concentration of the tested peptide required for half maximal inhibition of the binding of the indicator peptide.

T cell stimulatory capacity of α-gliadin derived peptides

Several of the amino acid variants that we found in the α-gliadin transcriptome have never been described before while some have been described but were never tested for their T cell stimulatory capacity. In order to determine which variants are capable of inducing T cell responses, the DQ2-Glia-α1, DQ2-Glia-α2, DQ2-Glia-α3, and DQ8-Glia-α1 variants were synthesized as 15- or 16-mer peptides and tested for their capacity to bind to HLA-DQ2 and induce *in vitro* proliferation of HLA DQ2- or DQ8-restricted T cell clones.

DQ2-Glia-α1 variants: virtually all peptides that carry the canonical 9-mer Glia-α1 epitope core $P_1\{F/Y\}_2P_3Q_4P_5E_6L_7P_8Y_9$ (in which the glutamic acid at p6 is introduced by TG2-deamidation of the original glutamine) were able to stimulate DQ2-Glia-α1 T cells. Only an arginine residue at the position preceding the epitope core diminished T cell stimulation. In the core sequence, several amino acid substitutions diminished or abolished the T cell stimulatory capacity, such as a proline to serine substitution at p3 or p8 and a proline to alanine substitution at p5 (Table 6, no. 3). Peptides in which an amino acid was deleted at p3 or p4 were not causing any proliferation of the T cells. Strikingly, such safe peptides are all from locus *Gli-B2* (Table 6, Figure S3).

DQ2-Glia-α2 variants: full responses of DQ2-Glia-α2 T cells were only observed against peptides that carry the core $P/F_1Q_2P_3E_4L_5P_6Y_7P_8Q_9$. A deletion of the glutamine at p2, indicative for α-gliadins from locus *Gli-B2,* or substitution of this glutamine by histidine diminished or abolished the stimulatory capacity. Furthermore, a single substitution of the proline for an serine residue at either p6 or p8 abolished the T cell stimulating capacity. The latter substitution is found in α-gliadins from locus *Gli-A2* (Table 6).

DQ2-Glia-α3 variants: also for this epitope several amino acid substitutions were found to destroy T cell stimulatory capacity, including an arginine to proline substitution at p2, which is found in α-gliadins from *Gli-B2* (Table 6).

DQ8-Glia-α1 variants: while several amino acid substitutions were found to influence T cell recognition of the canonical sequence $Q_1G_2S_3F_4Q_5P_6S_7Q_8Q_9$, a single serine to phenylalanine substitution at p3 and a single glutamine to arginine substitution at p5 were found to completely destroy T cell stimulatory properties (Table 6). While the former T cell stimulatory variants are found in α-gliadins from *Gli-D2* and *Gli-B2*, the glutamine to arginine variants are from *Gli-A2* (Table 5, Figure S2).

Thus, the α-gliadins encoded by *Gli-A2* of bread wheat are marked with a specific single amino acid substitution (P to S at p8) and lack the capacity to stimulate Glia-α2 T cell clones, whereas α-gliadins encoded by *Gli-B2* carry a deletion that prevents Glia-α1 T cell clone stimulation. Alpha-gliadins with the two intact epitopes, Glia-α1 and Glia-α2, are encoded by *Gli-D2* (Table 2 and 3).

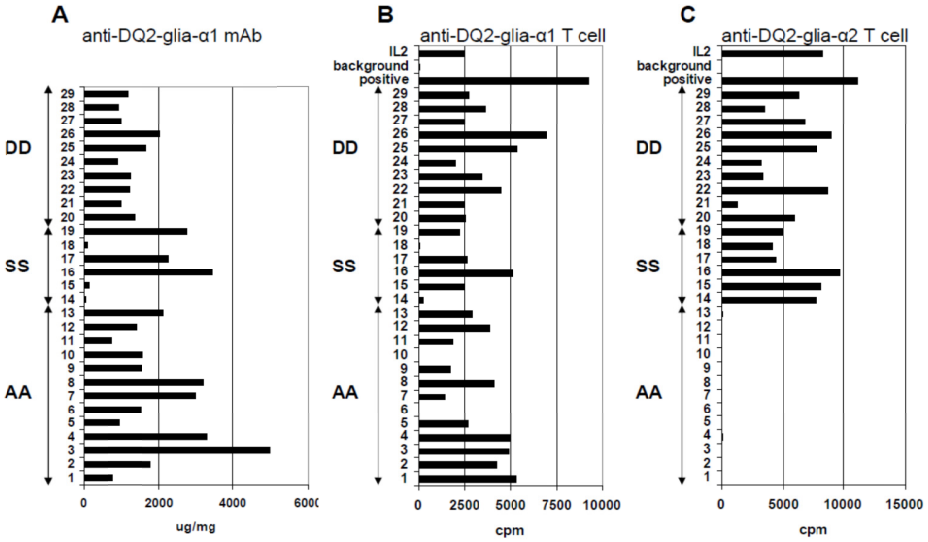

Figure 1. Presence of DQ2-Glia-α1 and DQ2-Glia-α2 epitopes in diploid wheat.

Pepsin-trypsin digests of 29 diploid wheat accessions were prepared and tested in a competition ELISA with a mAb specific for a sequence partially overlapping with the DQ2-Glia-α1 and DQ2-Glia-α2 epitopes and after deamidation with T cell clones specific for the DQ2-Glia-α1 and DQ2-Glia-α2 epitopes. A: results of the competiton ELISA. B: T cell proliferation assay with a DQ2-Glia-α1 specific T cell clone. C: T cell proliferation assay with a DQ2-Glia-α2 specific T cell clone. IL-2: proliferation of the T cell clone under the influence of interleukin-2. Background: proliferation of the T cells in the presence of antigen presenting cells but no antigen. Positive control: proliferation of the T cell clone in the presence of a synthetic peptide encoding the specific α-gliadin epitope and antigen presenting cells. cpm: counts per minute. AA: diploid accessions with an A genome; SS: diploid accessions with an S genome; DD: diploid accessions with a D genome.

Table S1. Alpha-gliadin transcripts of *T.monococcum* accessions.

T.monococcum accession	N_{total}	DQ2-Glia-α1/-α2/-α3 region	% of N_{total}
CGN10500	64	PQLQPFPSQQPYLQLQPFPQPQLPY**S**QPQPFRPQQPYPQPQPQYS PQ**P**QPFPSQQPYLQLQPFPQPQLPY**S**QPQPFRPQQPYPQPQPQYS	45%(29) 55%(35)
CGN12035	8	PQLQPFPSQQPYLQLQPFPQPQLPY**S**QPQPFRPQQPYPQPQPQYS	100%(8)
CGN10555	39	PQLQPFPSQQPYLQLQPFPQPQLPY**S**QPQPFRPQQPYPQPQPQYS PQ**P**QPFPSQQPYLQLQPFPQPQLPY**S**QPQPFRPQQPYPQPQPQYS	62%(24) 38%(15)

The amino acid sequence in the DQ2-Glia-α1/-α2/-α3 region (underlined), as deduced from counting's of more then four α-gliadin transcripts. The transcripts are derived from developing seeds of T.monococcum accessions. The natural proline (P) to serine (S) substitution at position 8 specific for α-gliadins from the Gli-A2 locus is depicted in red. N_{total}= total amount of transcript clones analyzed.

T cell stimulatory capacity of diploid wheat accessions

Previously, differential reactivity of α-gliadin specific T cell clones against gluten extracts from diploid wheat accessions has been reported [18,19]. In order to link such differential reactivity to the presence of specific epitope variants, a panel of diploid wheat accessions containing either the A, S or D genome was tested for their reactivity of an α-gliadin specific monoclonal antibody (mAb) and T cells.

Pepsin-trypsin digests of gluten extracts from kernels of 29 diploid *Triticum* and *Aegilops* accessions were prepared and tested in a competition ELISA with a mAb specific for a sequence partially overlapping with the DQ2-Glia-α1 and DQ2-Glia-α2 epitopes **(**Figure 1A**)** and, after treatment with TG2, with T cell clones specific for either the DQ2-Glia-α1 and DQ2-Glia-α2 epitope (Figures 1B and 1C). The results indicate that the mAb reacts strongly with all extracts except three derived from diploids expressing the S genome (Figure 1A). Similarly, and in agreement with previous results [18], extracts from A, S and D origin were capable of stimulating the DQ2-Glia-α1 specific T cell clone (Figure 1B) while the extracts of the diploids expressing the A genome failed to stimulate the DQ2-Glia-α2 specific T cell clone (Figure 1C).

Based on our observation that the α-gliadins expressed from locus *Gli-A2* of the T. *aestivum* A genome carry a variant DQ2-Glia-α2 epitope in which the proline at p8 has been replaced by a serine, and our experimental result that introducing this substitution in a peptide leads to loss of DQ2-Glia-α2 T cell stimulatory properties **(**Table 6), we wanted to determine if this amino acid substitution was indeed the cause of loss of immunogenicity. For this purpose we sequenced the α-gliadin locus of three diploid wheat (*T. monococcum*, A genome) accessions. In agreement with the results from hexaploid wheat transcripts (Figure S2) we observed that the α-gliadin transcripts from *T. monococcum* contain only a single form of DQ2-Glia-α1 and DQ2-Glia-α2. Moreover, in the DQ2-Glia-α2 epitope the proline at p8 was consistently replaced by a serine (Table S1). Together these results establish that this naturally occurring single amino acid substitution is sufficient to completely eliminate the T cell stimulatory properties of the DQ2-Glia-α2 epitope in gluten.

Differential reactivity of the DQ2-Glia-α3 specific T cells towards the extracts of the diploids correlated with an arginine to proline replacement at p2 in α-gliadins derived from the S-genome, FRPQQPYPQ→ FPPQQPYPQ (Table 6).

Elimination of α-gliadin toxicity by a naturally occurring single amino acid substitution

The large majority of known antigenic peptides derived from wheat gluten, as well as homologous peptides derived from the hordeins from barley and the secalins from rye, contain a proline at p8 [24]. Based on our observation that a single proline to serine substitution at p8 induced unresponsiveness of DQ2-Glia-α2 specific T cells and the previous observation that a similar substitution at p8 of the DQ2-Glia-α1 epitope induced T cell unresponsiveness (Table 6; peptide no.4), we investigated if a similar substitution would also eliminate the antigenic properties of the DQ2-Glia-α3 epitope. Wild type versions of the DQ2-Glia-α1, -α2 and -α3 epitopes as well as versions in which the proline at p8 was substituted by a serine were synthesized and tested in T

cell proliferation studies. As expected neither the substituted DQ2-Glia-α1 epitope (QLQPFPQPEL**S**YPQPQ) nor the substituted DQ2-Glia-α2 epitope (QLQPFPQPELPY**S**QPQ) induced T cell proliferation of respectively DQ2-Glia-α1 and DQ2-Glia-α2 specific T cells (Table 6**)**. Likewise, the substituted DQ2-Glia-α3 epitope failed to induce T cell activation (Figure 2A).

We also analysed the effects of proline to serine substitutions in the 33-mer α-gliadin derived peptide that encodes 6 partially overlapping antigenic DQ2-Glia-α1 and -α2 sequences (Figure 2B) as well as in an elongated version of the 33-mer which also encodes the DQ2-Glia-α3 epitope (Table S2). In all cases, the proline to serine substitutions completely abrogated the response of DQ2-Glia-α1, DQ2-Glia-α2 and DQ2-Glia-α3 specific T cell clones (Figure 2B, Table S2).

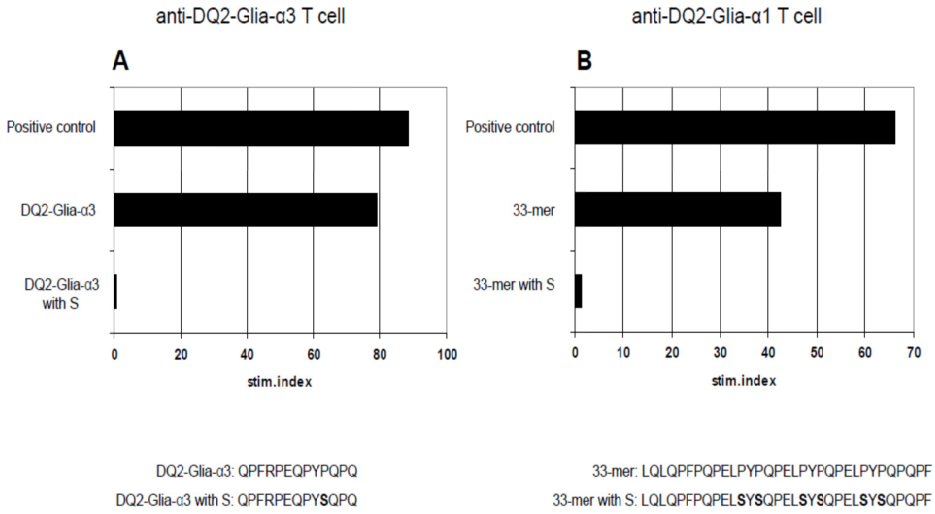

Figure 2. Amino acid substitution eliminates toxicity of known α-gliadin epitopes.

The DQ2-Glia-α3 epitope and the known 33-mer were synthesized in deamidated form either as the original sequence or after substitution in each epitope of the prolines at position 8 with serine. These peptides were tested in T cell proliferation assays. A: T cell proliferation assay using a DQ2-Glia-α3 T cell clone. B: T cell proliferation assay using a DQ2-Glia-α1 T cell clone. Positive control: synthetic peptide encoding the specific minimal T cell epitope. Stim. index: stimulation index defined as the specific proliferation of a sample divided by the background proliferation.

Table S2. The effects of proline to serine substitutions in an elongated version of the 33-mer, which also encodes the DQ2-Glia-α3 epitope.

Stimulator peptide	SI
DQ2-Glia-α3 eitope	59
DQ2-Glia-α3 substituted epitope	1
Elongated 33-mer epitope	49
Elongated substituted 33-mer epitope	1

Finally, we tested the effect of systematic introduction of single, double, triple and quadruple P to S substitutions in the DQ2-Glia-α-1 peptide with five T cell clones isolated from small intestinal biopsies of 3 CD patients. T cell responses of single T cell clones were only observed for two of the single P to S substitutions at p3 and p5. In contrast, with a single P to S substitution at p8 and in all but one of the other cases the substitutions completely eliminated the T cell response of all five T cell clones (Figure 3).

Thus, the naturally occurring proline to serine substitution constitutes a universal approach to remove the antigenic properties of HLA-DQ2 restricted α-gliadin peptides.

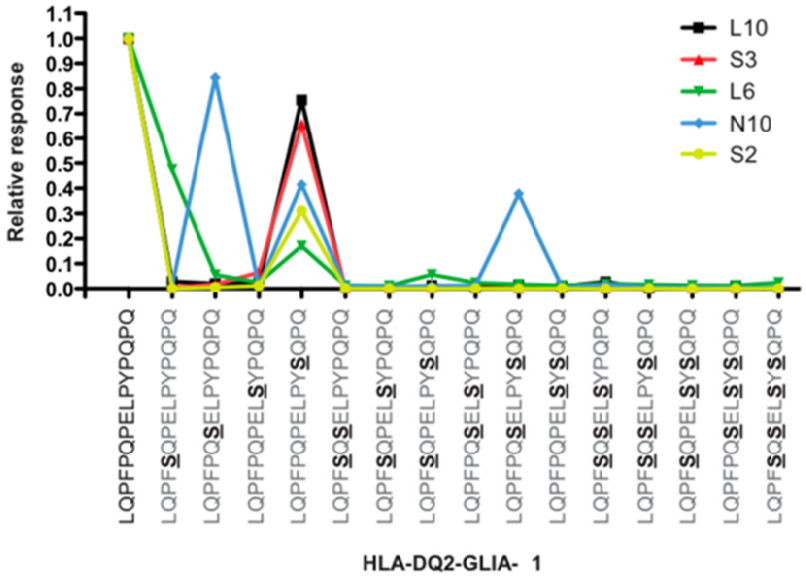

Figure 3. Response of DQ2-Glia-α1 epitope specific T cell clones against single and multiple P to S substituted peptides.

Five T cell clones derived from 3 CD patients were tested against the deamidated form of the DQ2-Glia-α1 peptide (sequence PFPQPELPY) and variants thereof in which prolines at position 3, 5, 8 and 10 were systematically substituted for serine, both as single substitutions and in all possible combinations. Shown is the response to the substituted peptides relative to unsubstituted DQ2-Glia-α1 epitope. The introduced substitutions are underlined. The most C-terminal substituted proline at position 10 lies outside the 9 amino acid core of the T cell stimulatory peptide.

DISCUSSION

Although T cell responses to peptides derived from α-, γ- and ω-gliadins as well as from HMW- and LMW-glutenins have been described, various studies have indicated that the α-gliadins are among the most immunogenic regarding CD [8-10,13,25]. A crucial step towards the elimination of gluten toxicity would thus be the elimination of T cell stimulatory α-gliadin sequences. Our extensive genetic analysis of over 3000 α-gliadin transcripts from different bread wheat accessions showed a high heterogeneity of the α-gliadins genes and considerable differences in the number of T cell stimulatory sequences encoded by the various α-gliadin genes. We identified three major factors determining these differences: i) the length of tandem repeats of antigenic sequences; ii) natural amino acid substitutions that affect the antigenicity of T cell epitopes, and iii) amino acid deletions that eliminate the antigenicity of T cell epitopes.

The α-gliadins from locus *Gli-D2* generally encode several copies of both the DQ2-Glia-α1 and DQ2-Glia-α2 epitopes in addition to the DQ2-Glia-α3 and DQ8-Glia-α1 epitopes, *Gli-A2* genes usually encode only the DQ2-Glia-α1 and DQ2-Glia-α3 epitopes while the *Gli-B2* genes encode no or at most one DQ2-Glia-α1 epitope, next to the DQ8-Glia-α1 epitopes. The 33-mer sequence with 6 T cell stimulatory sequences [13] was only found in a minority of the α-gliadins analyzed, all of which are expressed from *Gli-D2*. Many natural occuring amino acid substitutions affecting the antigenicity of the canonical α-gliadin peptides were identified. Typical examples are the proline to serine substitution at p8 in the DQ2-Glia-α2 epitope and the arginine to proline substitution at p2 in the DQ2-Glia-α3 epitope, that both completely eliminate the T cell stimulatory properties of these peptides.

The analysis of gluten extracts from the diploid wheat varieties underscores these observations and provides a molecular basis for the previous observation that A-genome diploid *T. monococcum* varieties lack the DQ2-Glia-α2 epitope [18]. All α-gliadins from this genome encode an altered version of the DQ2-Glia-α2 epitope with a serine at p8 that fails to induce T cell responses. We observed that a similar substitution eliminates the T cell stimulatory properties of the DQ2-Glia-α1 and DQ2-Glia-α3 epitopes. The more variable reaction pattern of T-cells and antibodies towards gluten extracts of the S-genomes reflects the higher level of genetic variation in these outcrossing species. These α-gliadins contain another variant of the DQ2-Glia-α1 and DQ2-Glia-α2 region that does not incude the A-genome specific serine at p8. Furthermore, amino acid deletions in the canonical α-gliadin peptides prohibit binding to HLA-DQ2 and hence T cell recognition. A typical example is the deletion of the glutamine at p4 in the DQ2-Glia-α1 epitope which generates a peptide that no longer binds to HLA-DQ2, presumably due to defective docking of the anchor residues into their respective pockets in the HLA-DQ2 molecule. Our results confirm previous observations [18, 19] that the α-gliadin locus *Gli-D2* encodes the most toxic α-gliadins while substantially less toxicity is associated with those from *Gli-A2* and *Gli-B2*, but also provide the molecular basis for these differences.

Unfortunately, due to the complexity of both the *Gli-2* gene family and the wheat genome, it will be a difficult task to generate tetraploid pasta and hexaploid bread wheat that is entirely safe for consumption by all CD patients by conventional breeding methods. Our results now provide a rationale for an alternative approach as we demonstrate that by the introduction of naturally occurring amino acid substitutions the toxicity of all four T cell epitopes in α-gliadins can be eliminated. Using novel methods such as zinc finger nucleases [26-28] we can introduce the underlying SNPs as specific mutations into the α-gliadin genes of wheat to eliminate toxicity completely. Technically this will not be easy, but our results indicate precisely the three complementary sets of actions that need to be performed.

i) In the *Gli-B2*-derived α-gliadins analyzed, none of the DQ2 epitopes are present due to a single amino acid deletion in the region encoding the DQ2-Glia-α1 and DQ2-Glia-α2 epitopes, which generates a peptide that has decreased binding affinity for HLA-DQ2 and is no longer recognized by T cells. Therefore, a single amino acid substitution in the DQ2-Glia-α3 epitope will result in a peptide that has completely lost HLA-DQ2 binding properties and T cell stimulatory properties. To eliminate the remaining DQ8-Glia-α1 epitope in some *Gli-B2* α-gliadins, a single glutamine to arginine substitution, which naturally occurs in the α-gliadins from the A-genome, would suffice. Such a minimally genetically modified B-genome α-gliadin gene would thus no longer encode any T cell stimulatory peptides. Moreover, by starting with an α-gliadin gene in which the sequence of the p31-43 peptide is naturally altered (for example the gene encoding protein no. 9 in SI 2), the chance of innate immune stimulation by a protein derived from such a gene would also be minimized.

ii) For *Gli-A2* α-gliadins, the approach to eliminate toxicity would be to introduce two proline to serine substitutions at p8 in the DQ2-Glia-α1 and DQ2-Glia-α3 epitopes present. As these α-gliadins genes encode a shorter version of the immunodominant 33-mer in which the DQ2-Glia-α-2 epitope is already non-functional, and contain a version of the DQ8-Glia-α1 epitope that has no T cell stimulatory properties, these two substitutions would completely remove toxicity in proteins encoded by such modified genes.

iii) Regarding the *Gli-D2* α-gliadins, we found that the proline to serine substitutions at p8 completely abrogated the in vitro T cell stimulatory properties of the 33-mer peptide and of an elongated version of the 33-mer that also encodes the DQ2-Glia-α3 epitope. This result underscores our previous observation that most T cell stimulatory gluten peptides have a proline at p8 [24]. However, to render α-gliadins from the D-genome non-toxic, up to seven substitutions need to be introduced in a single gene.

An alternative approach is to design safe α-gliadin genes that can subsequently be introduced into celiac disease safe cereals such as rice or maize, for the production of gluten proteins. As such modified proteins will be very similar to existing α-gliadins they will most likely have indistinguishable technological properties. Thus, such gluten proteins could enhance the baking properties of these cereal crops, or they could be extracted from these crops and used as an ingredient to generate novel high quality foods for celiac disease patients. For the generation of high quality cereals that can replace wheat-based products the simple introduction of detoxified α-gliadins is un-

likely to be sufficient, as baking quality is mostly determined by the HMW and LMW glutenin proteins. Therefore, additional studies will have to investigate how other gluten proteins can be detoxified as there is substantial evidence that these contain T cell stimulatory peptides as well. In previous studies we provided evidence that such epitopes can be found in the γ-gliadins as well as in the LMW- and HMW-glutenins [11, 12] and others have extended these observations [14, 25]. In particular, we found that T cell responses to LMW-glutenins were found in children while these are much less frequent in adults [12, 25]. Moreover, in a recent study a highly antigenic ω-gliadin peptide was described [25] that is identical to an antigenic hordein-derived peptide reported by us earlier [20]. In essence this hordein/omega peptide is a sequence variant of the DQ2-Glia-α1 peptide and also carries a proline at the p8 position [20]. It is therefore feasible that the toxicity of this peptide can be eliminated by a proline to serine substitution at p8 as well. Preliminary results show that amino acid substitutions similar to those that destroy the T cell stimulatory properties in α-gliadins might also be effective for γ-gliadin derived epitopes (Salentijn et al, in prep), but it is likely that for the LMW- and HMW-glutenins other approaches will be required. This will be the subject of further studies.

In conclusion, we have demonstrated that by utilizing naturally occurring amino acid substitutions the toxicity of the four T cell epitopes in α-gliadins can be eliminated. Such modified proteins will most likely display indistinguishable technological properties. Thus, our results provide a rational approach to eliminate CD related toxicity from α-gliadins, which represents a first but crucial step towards the realization of safe gluten containing food products for CD patients.

MATERIAL AND METHODS

Analysis of α-gliadin transcripts from diploid wheat varieties

T. monococcum accessions CGN10500, CGN12035 and CGN10555 (CGN, Wageningen, The Netherlands) were used for cloning and sequencing of α-gliadin transcripts. Plants were grown under greenhouse conditions. Developing green kernels of single plants were harvested and used for RNA isolation according to Doyle and Doyle [29] but with 1% (w/v) poly-(vinylpyrrolidone)-10 in the extraction buffer. For the production of first strand cDNA 1 µg of total RNA was treated with DNAse I (Invitrogen, amplification grade; 18068-015) followed by RT PCR (Invitrogen SuperScript III First-Strand Synthesis System for RT-PCR; 18080-051) using random hexamer primers in a final reaction volume of 20 µl. Primers specific for α-gliadin genes, located on the conserved sequences at the 5' and 3' end of the coding region of the α-gliadin, were used to amplify α-gliadin transcripts from the cDNA samples (αF1: 5'-atgaaRaCmtttcYcatc and α5R: 5'-gttagtaccgaNgatgcc). The PCR conditions: 5 min. at 94°C, 30 cycles (94°C for 1 min., 49°C for 1 min. and 72°C for 2 min), 72°C for 10 min, 25 µl reaction volume. The PCR products were cloned and sequenced.

Characterization of expressed α-gliadin sequences

Over 3200 *T. aestivum* expressed sequence tags (ESTs) and mRNAs designated as α-gliadin or α/β-gliadin were downloaded from the NCBI UniGene library (Ta.15268, Ta.23792, Ta.24084, Ta.25210, Ta.27702, Ta.28482) (http://www.ncbi.nml.nih.gov/UniGene) on 13 April 2007. The sequences were from *T. aestivum* libraries of various tissues, treatments and cultivars (Chinese Spring 34.2%; Glenlea 20.8%; Cheyenne 10.4%; Recital 7.8%; Mercia 7.2%; unknown cultivar 8.9%; Wyuna 3.2%; HiLine 2.9%; Butte 86, 2.4%; Hartog 1.5%; Soleil 0.6%; Nostar 0.1%; Novobirskaya 67, 0.1%). The DNA sequences were aligned using the SeqMan II (DNAstar) and first assembled at a minimum match percentage of 60%, gap lengths 3200, maximum match size 50bp. BLAST analysis of the contigs was performed to verify the α-gliadin identity of the contigs and short (<100bp) and bad sequences were discarded. The transcript contigs of ≥60% homologous sequences were trimmed up to the start and stop codons. Next, the sequences were reassembled at 90% homology, which resulted in 55 α-gliadin transcript contigs containing 1 to 475 sequences. The 3' end was covered by 50 contigs (2911 transcripts) whereas the 5' end was present in 30 contigs (2753 transcripts). The consensus of these contigs were saved in separate files and used for phylogenetic studies to deduce the genome of origin of the sequences in each contig.

Phylogenic analysis

With the aim to deduce the locus, *Gli-A2*, *Gli-B2* or *Gli-D2,* from which the transcripts were expressed, the 55 α-gliadin EST consensus nucleotide sequences obtained from clustering, were aligned using Clustal W, *MEGA* 4 [30], together with 56 genomic DNA sequences of known origin, i.e. derived from the diploid wheat species *T. monococcum* (A genome), *T. speltoides* (S genome) and *Aegilops tauschii* (D genome) [18] and DNA sequences that were previously assigned to a locus [31]. The sequences that covered the 5' region of the α-gliadin sequences were trimmed up to the start and up to nucleotides coding for the conserved amino acid motif PIS, located just in front of the first glutamine repeat, to cover the same region and subsequently used to generate a Neighbor-Joining tree (bootstrap test of 1000 replicates, pairwise deletion of gaps and missing data, Kimura 2-parameter, Substitutions to Include Transitions + Transversions; Pattern among Lineages Homogeneous, Uniform rates among sites, number of sites= 750, in MEGA 4) (Figure S1).

Sequence variation in epitope regions

To analyze all sequence variation, the 55 α-gliadin EST contigs, now assigned to a specific chromosome, were reassembled one by one at 99-100% match (SeqMan II, Lasergene, DNAstar). This yielded 717 different allelic variants. The consensus nucleotide sequences were translated (MEGA 4) and explored for epitopes and surrounding sequence regions using a text explorer program (PatternResearch, in house developed) after which the output file was analysed in Excel.

T cell clones, T cell proliferation and HLA-DQ2 binding assays

Gluten specific T cell clones were generated from small intestinal biopsies of celiac disease patients as described before [8,11,12]. All patients signed an informed consent form which was approved by the hospital ethics committee. Proliferation assays were performed in triplicate in 150 µl Iscove's Modified Dulbecco's Medium (Bio Whittaker, Verviers, Belgium) with 10% pooled normal human serum in 96 well flat-bottom plates using 2x104 gluten specific T cells stimulated with 105 irradiated HLA-DQ2-matched allogeneic peripheral blood mononuclear cells (3000 rad) in the presence or absence of antigen (1-10 µg/ml) [8, 11, 12]. After 2 days ^{3}H-thymidine (0.5 µCi/well) was added to the cultures, and 18-20 hours thereafter the cells were harvested. ^{3}H-thymidine incorporation in the T cell DNA was determined with a liquid scintillation counter (1205 Betaplate Liquid Scintillation Counter, LKB Instruments, Gaithersburg, MD)**.** A binding assay was performed as described previously [24].

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Chapter 4

Natural variation in avenin epitopes among oat varieties: implications for Celiac Disease

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ABSTRACT

Celiac disease (CD) is a chronic inflammatory disease affecting the small intestinal mucosa. The causative agents have been identified as gluten proteins from wheat, barley and rye, and the only available treatment for CD patients is a lifelong gluten-free diet. Non-gluten containing cereals would be a valuable contribution to the gluten-free diet. In this respect, oats are a good choice. However, commercial lots of oat flakes and flour frequently are contaminated with wheat, barley and rye, and two studies have reported that some peptides derived from the gluten-like avenin storage proteins of oat can trigger an immune response in some CD patients. In the present study we have initiated the investigation whether all oat varieties contain similar amounts of potentially harmful sequences by biochemical and immunological methods. We confirm that commercial oat preparations are contaminated with other cereals that contain gluten or gluten-like proteins. Moreover, our results demonstrate that contamination-free oat varieties differ in their capacity to stimulate an avenin-sensitive gamma-gliadin specific T cell line derived from a patient with CD, indicative for differences in the two known avenin epitopes among oat varieties, implying that selection and breeding of completely safe oat varieties for all CD patients may be a realistic possibility.

INTRODUCTION

Celiac disease (CD) is a food intolerance that affects approximately 1% of the population (10). Typical symptoms include diarrhea, abdominal distention and pain. Extra-intestinal manifestations like anemia, infertility, growth deficiency and neurological symptoms can also be present (4).

CD is an immune mediated disease in which protein fragments from wheat, barley and rye provoke an inappropriate immune response. It is well established that the disease almost only develops in HLA-DQ2 and/or -DQ8 positive individuals (11, 19). HLA-DQ2 and -DQ8 are HLA-class II molecules involved in binding peptides derived from exogenous proteins and "presenting" these peptides to the T cells of the immune system (9). Both HLA-DQ2 and -DQ8 can bind gluten-derived peptides, particularly after enzymatic modification by the enzyme tissue transglutaminase (tTG) (2), which introduces negative charges in gluten peptides required for efficient binding to the HLA-molecules. Upon binding, the HLA-DQ-gluten peptide complexes can trigger inflammatory T cell responses which ultimately lead to disease (14, 28). As such gluten specific T cells can only be isolated from the small intestine of CD patients, these adaptive immune responses are a critical factor in disease pathogenesis.

Upon withdrawal of gluten the inflammation subdues and patients can lead a normal life as long as they stick to a lifelong gluten-free diet, thus devoid of any products prepared from wheat, barley and rye. Food products based on gluten-containing cereals, however, form an important component of the human diet and celiac patients need alternative cereals that substitute this source of fiber and nutrients. One of the possible candidates is oat but this is still controversial as contradictory reports have appeared concerning the safety of oat for CD patients. Several studies have documented that >99% of CD patients can safely consume oat (6, 7, 15), and on that basis non-contaminated ('pure') oat is now considered as gluten-free in EC-regulation 41/2009.

However, two studies have found CD patients that do not tolerate contamination-free oats: three CD patients developed intestinal inflammation upon oat exposure (1) and one developed partial villous atrophy (8). It has also been demonstrated that gluten-reactive T cells from some CD patients can also respond to avenin-derived peptides (24). Also, an avenin specific T cell line has been isolated from the biopsy of a celiac patient which developed villous atrophy during an oat-containing, but otherwise standard gluten-free diet (1). These results thus indicate that oat may not be completely harmless to all patients. Furthermore, contamination of oats with other cereals, due to the shared use of equipment for transportation and fabrication for both oat and other cereals, is quite frequent as was previously reported (3, 5). Therefore, the toxicity of oat can be due to both contamination and intrinsic toxicity but the result is the same: it leads to uncertainty about introduction of oat in the gluten-free diet, especially in those countries where oat is a not-frequently consumed food product.

The present study is focused on the characterization of this potential intrinsic immunogenicity of a selection of 26 oat varieties using immunological and biochemical methods.

MATERIALS AND METHODS

Oat samples

The grains of twenty-six oat varieties (1: Ascot, 2: Astor, 3: Charming, 4: Charmoise, 5: Dalguise, 6: Dominik, 7: Fervente, 8: Firth, 9: Freddy, 10: Gambo, 11: Gele van Timmermans, 12: Gerald, 13: Gigant, 14: Leanda, 15: Mansholt III, 16: Markant, 17: Mustang, 18: Ouderwetse Zeeuwse Partij, 19: Panache de Roye, 20: Powys, 21: Sang, 22: Troshaver uit Besel, 23: Valiant, 24: Wodan, 25: Zandster, 26: Zwarte President) were used in this study. All varieties were obtained from CGN (Wageningen, The Netherlands) and the grains were washed with 60% aqueous ethanol and dried over-night to remove any trace of other cereals before grinding in a coffee mill to obtain a fine homogenized powder. As contamination of oats by other cereals is well documented (3, 5), we analyzed eight varieties for possible contamination using a sandwich R5 ELISA kit (Ingezim® Gluten, Ingenasa, Spain) and a competition assay based on a specific mAb which recognizes the α20-gliadin epitope and homologous sequences from barley and rye (20). These varieties were Astor, Gele van Timmerman, Mansholt III, Mustang, Panache de Roye, Troshaver uit Besel, Wodan and Zwarte President. All samples were found to be contamination free by both methods (< 1.5 mg/kg for the R5 method and < 25 µg/kg for the α20-gliadin epitope specific competition assay).

Preparation of protein fractions from oat varieties

Prolamins were extracted from the oat samples using 60% (v/v) ethanol as described before (3). Trypsin/pepsin digests were prepared as follows: 0.5 g of oat sample was solubilized in 4 mL of 1 mol/L acetic acid and boiled for 15 minutes. After cooling to room temperature (RT) 2.5 mg of pepsin was added and the mixture was incubated for 4 hours at 37° C. Subsequently the pH was adjusted to 7.8 with NaOH, followed by addition of 5 mg of trypsin. After incubation overnight at 37°C the samples were boiled for 15 minutes. For the next 48 hours the samples were dialyzed against water using dialysis tubing with a cutoff of 10 kDa. The dialyzed material was centrifuged and fractionated over a 10 kDa membrane for removal of the enzymes and any remaining insoluble material. For the subsequent experiments the fractions smaller than 10 kDa were used. A control sample was prepared using a commercial available gliadin preparation. For the T-cell assay the pepsin/trypsin digests were treated with tissue transglutaminase (N-Zyme) as described previously (22).

T cell proliferation assays

The presence of T cell stimulatory epitopes in the oat samples was determined using a T cell line isolated from a small intestinal biopsy of a celiac disease patient (24). Proliferation experiments were performed in triplicate in 150 µL Iscove's Modified Dulbecco's Media (IMDM) with 10% normal human serum in 96-well flat-bottom plates using $2x10^4$ gluten specific T cells stimulated with 10^5 irradiated (3000 rad) HLA-DQ2 or -DQ8 matched allogenic peripheral blood mononuclear cells (PBMCs) in the presence of or absence of the antigen (4 µg/well). After 2 days 0.5 µCi/well ^{3}H-thymidine was added to the cultures and after 18-20 hours the cells were harvested and the ^{3}H-tymidine incorporation was measured using a liquid scintillation counter (MicroBeta counter, Perkin Elmer).

Synthetic peptides

Peptides were synthesized by standard Fmoc chemistry on a SyroII peptide synthesizer as described previously (20). The integrity of the peptides was checked by reversed-phase HPLC and mass spectrometry. When required, biotin was introduced in the resin-bound peptides by a 2-h coupling with a 6-fold equimolar preactivating mixture of biotin and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate.

Protein analysis by 1D sodiumdodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

To determine the level of T cell stimulatory epitopes, 10 µL of the prolamin extracts were dried in a CHRIST ALPHA freeze-dryer (Salm en Kipp, Breukelen, The Netherlands), resuspended in 20 µL of protein sample buffer [62.5 mM Tris-HCl pH 6.8, 5% (v/v) glycerol, 2% (w/v) SDS, 0.0005% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol] and incubated for 5 min at 95ºC in a water bath. After that, the samples were spin down using a centrifuge and 20 µL supernatant was loaded into the wells of a 12.5% (w/v) SDS-PAGE gel. The proteins were visualized either directly using Imperial™ Protein Stain (Pierce, Rockford, IL) or transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). For the Western Blot analysis, the proteins were visualized with monoclonal antibodies (mAbs) specific for stimulatory T cell epitopes from α20-gliadin and Low Molecular Weight (LMW)-glutenin (12, 20, 21).

Competition assay for the quantitative detection of a T cell stimulatory epitope

The content of a T cell stimulatory epitope involved in celiac disease and present in α20-gliadin was determined using specific Enzyme-Linked ImmunoSorbent Assays (ELISAs). Maxisorb Immunoplates (Nunc, Copenhagen, Denmark) were coated overnight at +4°C with 100 µL/well of 2-5 µg/mL mAb in Phosphate Buffered Saline (PBS; 154 mmol/L NaCl and 1.4 mmol/L NaH_2PO_4/Na_2HPO_4; pH 7.5). The plates were washed (5 times) with 0.02% (v/v) Tween-20 in PBS and the residual binding sites on the plates were blocked for 30 min at RT with 150 µL/well of 2% (w/v) skim milk powder (Fluka, Zwijndrecht, The Netherlands) in PBS. After a washing step, the plates were incubated for 1 h at RT with 50 µL/well of different dilutions of the prolamin extracts with 0.1%

(v/w) Tween-20/0.2% (w/v) skim milk powder in PBS, mixed with another 50 µL/well of the biotinylated indicator peptide at a concentration of 0.002-10 µg/mL, also with 0.1% (v/w) Tween-20/0.2% (w/v) skim milk powder in PBS. After this step, the plates were washed and incubated for 30 min at RT with an excess of streptavidin-conjugated horseradish peroxidase (Sigma Aldrich, Zwijndrecht, The Netherlands) diluted with 0.2% (w/v) skim milk powder in PBS. After a washing step, bound peroxidase was visualized by incubation for 30 min, at RT and darkness, with 100 µL/well of a solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Aldrich). The color reaction was stopped by the addition of 100 µL/well of 2 M H_2SO_4. Finally, absorbance at 450 nm was read on a multiscan plate reader (Wallac, Turku, Finland). For quantification, the standard curve was made using a synthetic peptide containing the immuno-stimulatory celiac disease epitope, in a concentration range from 1 µg/mL to 1 ng/mL. The assays were repeated at least twice.

Direct binding assay

Direct binding assays were performed in a similar way as the competition assays for the quantitative detection of T-cell stimulatory epitopes (a20-gliadin and LMW-glutenin). Maxisorb Immunoplates (Nunc, Copenhagen, Denmark) were coated overnight at +4°C with 100 µL/well of the different peptides in a concentration range between 0.1 and 10 µg/mL in Phosphate Buffered Saline (PBS; 154 mmol/L NaCl and 1.4 mmol/L NaH_2PO_4/Na_2HPO_4; pH 7.5). The plates were washed (5 times) with 0.02% (v/v) Tween-20 in PBS and the residual binding sites on the plates were blocked for 30 min at RT with 150 µL/well of 2% (w/v) skim milk powder (Fluka, Zwijndrecht, The Netherlands) in PBS. After a washing step, the plates were incubated for 1 h at RT with 100 µL/well of the different mAbs at a concentration of 1.5 µg/mL with 0.1% (v/w) Tween-20/0.2% (w/v) skim milk powder in PBS. After this step, the plates were washed and incubated for 30 min at RT with an excess of rat-anti-mouse horseradish peroxidase conjugated polyclonal antibodies (Sigma Aldrich) diluted with 0.2% (w/v) skim milk powder in PBS.

In gel digestion and characterization of proteins by mass spectrometry

The desired gel bands, isolated from an Imperial™ (Pierce, Rockford, IL) stained gel, were digested with chymotrypsin using the Proteineer DP digestion robot (Bruker, Bremen, Germany). The protocol supplied by the manufacturer was followed. Digested proteins were analyzed by mass spectrometry as described previously (23). Searches were performed in the UniProt kB database by using FASTA alignment as described previously (23).

RESULTS

A gamma-gliadin specific T cell line differentially responds to oat samples

Previously we have shown that a gamma-gliadin specific T cell line isolated from a small intestinal biopsy of a child with CD was reactive with avenin-derived peptides (24). We now used this T cell line to test for the presence of such peptides in the selection of oat samples. For this purpose pepsin/trypsin digests were prepared and treated with tTG. As controls we included four avenin-derived peptides, two of which were shown to stimulate the T cell line (24). Subsequently these samples were tested for T cell stimulatory capacity in a T cell proliferation assay. The results demonstrate that the majority of oat samples contain epitopes that can stimulate the gamma-gliadin specific T cell line, largely similar to the stimulation by the control avenin peptides but less strong compared to stimulation with gliadin. However, several samples were found to hardly induce T cell proliferation, indicating that some varieties appear to contain a substantial lower amount of T cell stimulatory avenin peptides (Fig. 1).

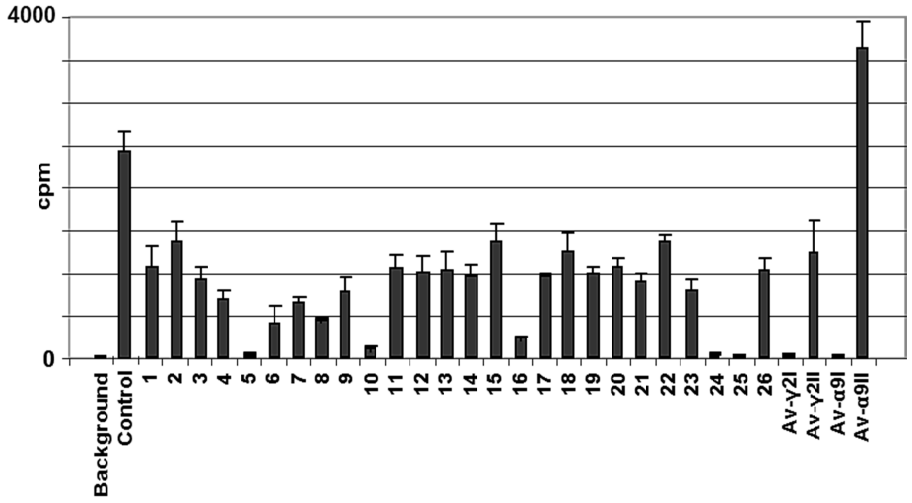

Figure 1. Proliferation assay using a gliadin specific T cell line known to recognize avenin epitopes.

Proliferation of a gamma-gliadin specific T cell line, in the presence of peptic/tryptic digests of 26 oat varieties and 4 synthetic peptides containing avenin epitopes. All samples were first treated with tTG and after measuring protein concentration identical amounts of the digests were used. The proliferative responses were measured by thymidine (^{3}H) incorporation. Background: proliferation in the absence of samples, control: proliferation in the presence of gliadin extracts, cpm: counts per minute. The experiment shown is representative of three independent experiments.

Anti-LMW-glutenin antibody cross-reacts with oat protein extracts

mAbs can be useful tools to screen for the presence of gluten or gluten-like molecules in cereals. In a previous study we have characterized the reactivity of several of such antibodies against wheat, barley, rye and oats. In addition we have generated a mAb for a LMW-glutenin derived peptide. This antibody specifically detects the sequence PFSQ, a sequence that shares homology with PFVQ which has a two-amino acid overlap with the avenin-derived T cell stimulatory peptides (24). We tested if this antibody would be useful for the detection of potentially harmful sequences in oat. First we determined if the antibody reacted with synthetic avenin peptides and observed binding of the antibody to the 4 avenin peptides tested but not to a control peptide lacking the PFVQ sequence (Fig. 2). Moreover, none of these peptides were recognized by the α20-gliadin specific mAb (results not shown). Subsequently we tested the reactivity of the antibody in a competition assay and in Western Blot analysis of the oat preparations. Strong reactivity was observed in the competition assay (results not shown) and in the Western Blot analysis (Fig. 3). To demonstrate that these detected proteins indeed correspond to the avenins, gel slices containing the detected bands were excised, treated with chymotrypsin and the resulting fragments were characterized by tandem mass spectrometry in combination with data base searches. This revealed that the detected bands contained two known avenin epitopes, both containing the PFVQ sequence: QQPFVQQQQPFVQQ and QYQPYPEQQQPFVQ. Together these results indicate that the mAb is able to detect avenin sequences that may contain an avenin epitope.

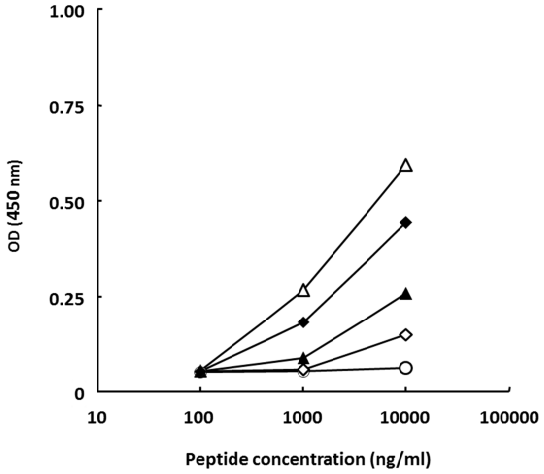

Figure 2. Reactivity of the anti-LMW-glutenin mAb against synthetic peptides containing avenin epitopes.

The reactivity of the anti-LMW-glutenin mAb against peptides possessing 4 known avenin epitopes was tested in direct binding assays. (△) Av-γ2I QQPFVQQQQPFVQ, (▲) Av-γ2II QQPFVQQQQPFVQQ, (◇) Av-α9I QYQPYPEQQEPFVQ, (◆) Av-α9II QYQPYPEQQQPFVQ and (○) control peptide QPGQGQPGYYPTSPQB. OD: optical density.

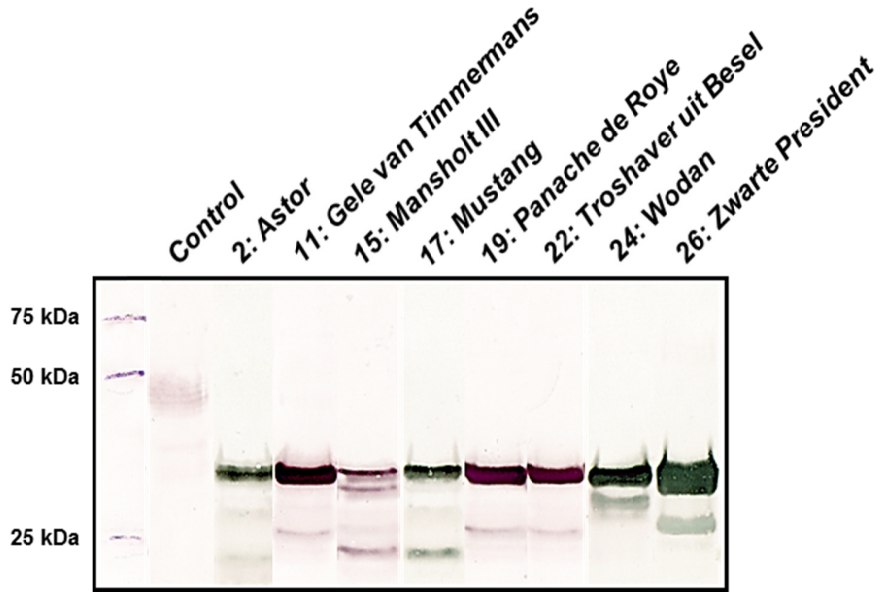

Figure 3. Western Blot analysis of 8 oat varieties.

Ethanol extracts were performed as described and same amount of proteins were separated on 1D-gel electrophoresis followed by Western Blotting and staining with an antibody recognizing the Glt-156 LMW epitope. The stained bands were isolated from the gel and digested for mass spectrometric analysis. Control: Rice baby food spiked with 1% (w/w) wheat baby food.

DISCUSSION

Since CD patients do not tolerate wheat, barley and rye, alternatives to substitute for these commonly used cereals are desirable. In this respect oat has been proven to be a good candidate, especially in the Nordic countries during the last decade (6), but the introduction of oat in the gluten-free diet, especially in those countries where oat is not commonly consumed, is still controversial as oat contains gluten-like avenins that are known to contain a few peptides that can stimulate T cells isolated from small intestinal biopsies of CD patients. Nonetheless, many reports have shown that CD patients can tolerate an oat containing gluten-free diet (6, 7, 15). There may, however, be a logical explanation for these seemingly contradictory findings: oat is phylogenetically more distantly related to wheat than barley and rye and this is reflected in several key differences between the avenins of oat and the gluten and gluten-like molecules in the other cereals. First, the amount of avenins in oat grains is substantially lower than that of gliadin in wheat (10% of the total protein content in oat, compared to 40-50% in wheat), as most storage proteins in oat are globulins. Second, avenins contain

less proline, the amino acid that contributes especially to the resistance of gluten to degradation in the gastrointestinal tract and that is crucial for the specific modification by the enzyme tTG that is linked to gluten toxicity (24). Hence, the potential of releasing stimulatory peptides is much lower. Together this means that oat contains much fewer sequences that are harmful for CD patients, and these are less abundant and more easily degraded in the gastrointestinal tract. This combination likely contributes significantly to the observed tolerance of CD patients to oat. It also fits in the "threshold model" in which a certain amount of exposure to immunogenic gluten peptides is tolerated while higher exposure leads to disease (24). Recent clinical data indicate that the addition of oat to a gluten-free diet can even result in more rapid intestinal improvement (Markku Mäki, personal communication, 2010).

A specific problem with oat is contamination (3, 5). Also in the present study did we observe that commercially grinded oat flours were mostly contaminated with other cereals (data not shown). Such contaminated oat is not considered safe for consumption by CD patients. This result underlines the importance of establishing a contamination-free oat chain for the production of suitable products for CD patients, which has been realized in Scandinavian countries and The Netherlands.

For the determination of the relative immunogenicity of the oat samples we made use of a gamma-gliadin reactive T cell line that was previously shown to also respond to two avenin peptides (24). Such gluten-reactive T cells are isolated from the small intestine of celiac patients and thus are strongly linked with the disease. Such cells can give valuable information on the immunogenicity of a sample. In a series of experiments with this T cell line we observed reproducible differences in the T cell stimulatory capacity of the oat samples, indicative of differences in immunogenicity among 26 oat varieties tested. This confirms and extends the results of Silano and collaborators, who observed differences among four oat varieties (17). A mAb originally raised against a LMW-glutenin peptide reacted with the oat preparations in Western Blot analysis, and mass spectrometric analysis demonstrated that the protein bands stained by the antibody contained, amongst others, two known immunogenic avenin peptides with sequence QQPFVQQQQPFVQQ and QYQPYPEQQQPFVQ.

The oat varieties gave different signals with the LMW-glutenin antibody and the gamma-gliadin reactive T cell line. For example, variety Wodan (#24), which showed a strong reaction against the LMW-glutenin antibody, was also found to hardly induce T cell proliferation. By contrast, variety Mansholt III (#15), which gave a faint Western Blot reaction, proved to be very active in stimulating the gamma-gliadin specific T cell line. These observations could be explained by the different recognition patterns of both systems as the LMW-glutenin antibody reacted with the four avenin peptides tested while the gamma-gliadin specific T cell line only responded to two of them. Since the T cells are isolated from patients, their reactivity pattern probably best reflects the situation *in vivo*.

In conclusion, our results show that most non-contaminated oat varieties contain avenin epitopes that are potentially harmful for a minority of the CD patient population. Similarly to the situation in wheat (16, 21, 26, 27, 29), not all oat varieties display the same immunogenic profile, suggesting that the selection and breeding of oat varieties that have a lower risk profile or have no risk at all for CD patients may be realistic.

A first step will be to clone and sequence avenin genes and cDNAs from oat varieties to analyze the presence of avenins with and without the epitopes. The T cell line will be useful to detect differences in the immunogenic potential of the oat material (flakes, flour, etc) derived from selected varieties. However, as true safety of cereals can only be ascertained when consumption by patients does not lead to clinical symptoms, further research is needed to determine the clinical relevance of our results.

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Chapter 5

Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: implications for celiac disease

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ABSTRACT

Celiac disease is caused by an immune response to gluten. As gluten proteins are proline-rich they are resistant to enzymatic digestion in the gastrointestinal tract, a property that likely contributes to the immunogenic nature of gluten. In this study we have determined the efficiency of gluten degradation by a post- proline cutting enzyme, prolyl endoprotease from *Aspergillus niger* (AN-PEP), in a dynamic system that closely mimics the human gastrointestinal tract (TIM-system). Two experiments were performed. In the first, a slice of bread was processed in the TIM system with and without co-administration of AN-PEP. In the second, a standard fast food menu was used. Samples of the digesting meals were taken from the stomach, duodenum, jejunum and ileum compartments at time zero until four hours after the start of the experiment. In these samples the levels of immunogenic peptides from gliadins and glutenins were assessed by monoclonal antibody based competition assays, Western blot analysis and proliferation T-cell assays. AN-PEP accelerated the degradation of gluten in the stomach compartment to such an extent that hardly any gluten reached the duodenum compartment. AN-PEP is capable of accelerating the degradation of gluten in a gastrointestinal system that closely mimics *in vivo* digestion. This implies that co-administration of AN-PEP with a gluten containing meal might eliminate gluten toxicity, thus offering patients the possibility to (occasionally) abandon their strict gluten-free diet.

INTRODUCTION

Celiac disease (CD) is a small intestinal disorder characterized by an abnormal immune response to gluten proteins. In CD patients ingestion of gluten evokes an immune response in the small intestine that eventually results in T cell infiltration and flattening of the mucosa (1). Patients experience malabsorption, diarrhea and failure to thrive, leading to fatigue, osteoporosis and/or neurological symptoms. Gluten proteins are the storage proteins of wheat and contain high percentages of proline (20%) and glutamine residues (38%). Because of their unusual high content of proline (2) gluten is poorly degraded by enzymes present in the gastrointestinal tract (GI-tract). After ingestion, partially degraded gluten proteins reach the small intestine. Such fragments are good substrates for the enzyme tissue transglutaminase which can convert the amino acid glutamine in gluten into the negatively charged glutamic acid. These modified gluten fragments can bind with high affinity to the disease-associated HLA-DQ2 or HLA-DQ8 molecules and induce inflammatory T cell responses (3-6).

Gluten is composed of two different protein families, the gliadins and glutenins. The gliadins can be further subdivided in α-, β-, γ-, and ω-gliadins and the glutenins into low molecular weight (LMW-) and high molecular weight (HMW-) glutenins. T cell stimulatory epitopes have been identified in all these proteins (7-13),(8;14;15).

For patients with CD the only cure is a lifelong strict gluten-free diet (GFD), which in practice excludes all foods that contain wheat, barley, rye and oats. Because of the widespread use of gluten and gluten-like proteins in food products, a GFD has a great impact on the lifestyle of CD patients. For this reason the search for new treatments, which are compatible with a normal social lifestyle, is of great importance. In this respect several lines of research have been proposed. Studies are performed in which wheat varieties are screened for the level of T cell stimulatory epitopes. Wheat varieties with a low toxicity might form the basis for future breeding programs to generate wheat varieties suitable for generation of food products that can be consumed by CD patients (16-18). Another option is the use of enzymes that degrade the proline-rich gluten molecules before they reach the small intestine. In this context prolyl oligopeptidases were investigated. Such enzymes are not only effective in degrading gluten, the generation of smaller gluten fragments also improves the digestibility of gluten proteins by rendering them more accessible to brush border enzymes (19-25). However, to avoid T cell recognition gluten must be degraded before it reaches the small intestine and the prolyl oligopeptidases investigated are not active under the conditions found in the stomach. These enzymes are thus not suitable for oral supplementation as an alternative treatment for CD.

Recently, we described a prolyl endoprotease from *Aspergillus niger* (AN-PEP) (39). This enzyme was found to efficiently degrade gluten peptides and intact gluten proteins. Moreover, the pH optimum of the enzyme is compatible with that found in the stomach and the enzyme is resistant to degradation by pepsin. These results indicate that this enzyme might be suitable for oral supplementation to degrade gluten proteins in food before they reach the small intestine. To test this we determined the

efficiency of gluten degradation under near *in vivo* conditions. To predict the efficacy of enzymes and drugs for therapeutic use in the GI-tract of humans, a dynamic, multi-compartimental *in vitro* system was developed (26). Validation studies demonstrated that this system, called TIM (TNO gastro-Intestinal Model), allows a close simulation of *in vivo* dynamic physiological processes that occur within the lumen of the stomach and small intestine of humans and reliably predicts *in vivo* data (27-29). The system is fully computerized and based on parameters obtained from data of healthy volunteers. The main parameters of digestion, such as pH, body temperature, peristaltic mixing and transit, salivary, gastric, biliary, and pancreatic secretions, as well as absorption of small molecules (e.g. nutrients and drugs) and water are simulated. GI passage and successive conditions can be adjusted in order to mimic parameters in humans at different stages (infant, adult, and elderly), different food intakes and physiological or pathological conditions (such as gastric hyperacidity or pancreatic failure) (29;30).

The result of the present study demonstrates that gluten degradation was strongly accelerated by the presence of AN-PEP in the stomach compartment. AN-PEP was capable of degrading all T cell stimulatory epitopes of gluten tested for to levels below the detection limit of the methods used. Co-administration of AN-PEP with a gluten containing meal may thus be a feasible approach to detoxify gluten before it can do harm in the small intestine of CD patients. This may offer patients an alternative to the strict GFD and thereby improve their quality of life.

MATERIALS AND METHODS

Enzyme prolyl endoprotease from *Aspergillus niger* (AN-PEP)

Prolyl endoprotease from *Aspergillus niger* (AN-PEP) was produced and purified by DSM Food Specialties (Delft, The Netherlands) according to established procedures (31). Activity of the protein is expressed in Proline Protease Units (PPU). A PPU is defined as the quantity of enzyme that releases 1 µmol of p-nitroanilide per minute at 37^0 C in a citrate/disodium phosphate buffer pH 4.6 and at a substrate concentration of 0.37 mM Z-Gly-Pro-pNA (Bachem, Bubendorf, Switserland) and represents 10 mg of pure protein. The reaction products were monitored spectrophotometrically at 405 nM.

TIM experiments

The TIM has been described in detail previously (26-30). This model has compartments for the stomach, duodenum, jejunum and ileum (Figure 1). Each compartment has a flexible inner wall surrounded by water at 37º C. Changing water pressure squeezes the walls to simulate peristaltic mixing of the food with the 'secreted' electrolytes and enzymes. The transport of the chyme is regulated by the peristaltic valves that connect the successive compartments. Using various sensors in the compartments, the pH values, temperature, volumes, and pressure, as well as the gastric emptying and small intestinal passage of the food are computer-controlled according to pre-set curves. For

the present experiments all parameters in TIM were adjusted to simulate the average physiological conditions in the GI tract of young healthy adults after the intake of the type of meal as described below. During 2.5 h the gastric content was gradually delivered into the small intestine via the 'pyloric valve'. After 5 h approximately 80% of the small-intestinal content was gradually delivered into the 'large intestine' (sampling bottle) via the 'ileo-caecal valve'.

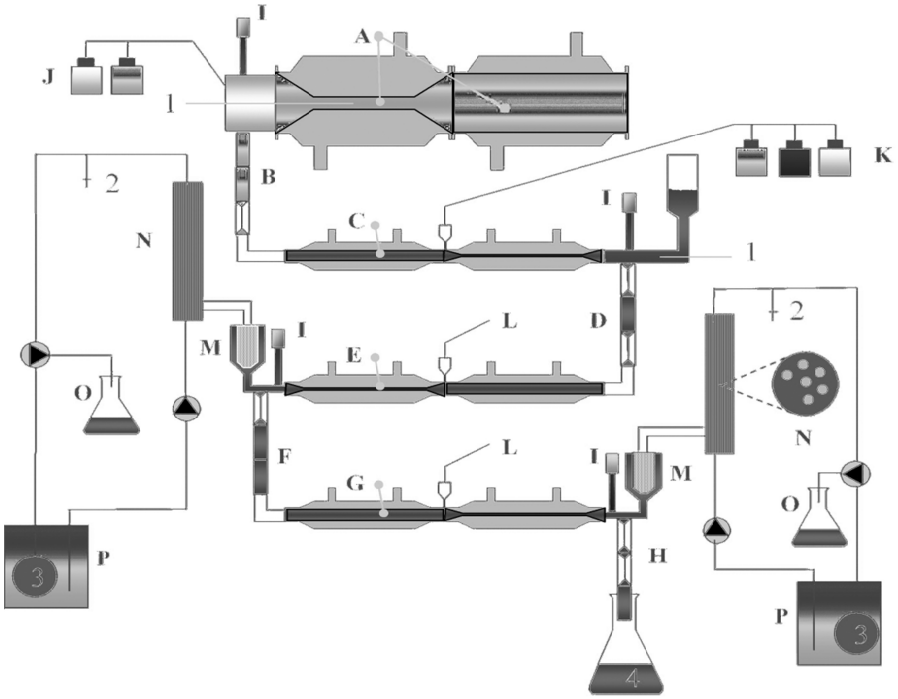

Figure 1. Schematic diagram of the dynamic, multi-compartmental model of the stomach and small intestine (TIM system).

A. stomach compartment; B. pyloric sphincter; C. duodenum compartment; D. peristaltic valve; E. jejunum compartment; F. peristaltic valve; G. ileum compartment; H. ileo-caecal sphincter; I. pH electrodes; J. pancreatin, bicarbonate; L. secretion of bicarbonate to control the intestinal pH; M. pre-filter system; N. hollow fibre semi-permeable membrane system; O. water absorption system; P. closed dialysing system.
For 2.5 hours the gastric content was mixed by peristaltic movements with added saliva and gastric juice (NaCl (4.8 g/L), KCl (2.2 g/L), CaCl2 (0.22 g/L) and NaHCO3 (1.5 g/L) with pepsin 500 KU/L (Sigma, P-7012) and gradually delivered into the small intestine via the 'pyloric valve'. Throughout the transit in the duodenum compartment the content was mixed by peristaltic movements with bicarbonate up to pH 6.4 ± 0.2 and with 'secreted' bile and pancreatic juice. Also in the jejunum and ileum compartments the content was mixed with bicarbonate up to pH 6.8 ± 0.2 and pH 7.1 ± 0.2, respectively. The digested compounds were dialyzed continuously from the jejunum and ileum compartments via semi-permeable hollow fiber membrane systems.

Two experiments were performed. In the first experiment 70 g of white bread (containing 5 g of gluten) and 110 ml drinking water was homogenized together with 110 ml of artificial saliva in the absence or presence of AN-PEP (200 mg pure enzyme/g protein). After 40 seconds of homogenization the mixture was added to the stomach compartment of the TIM-system containing 10 ml of simulated gastric juice and the experiment was started. In the second experiment a quarter of a commercial fast food menu consisting of a bread bun, a hamburger, ketchup, French fries, and supplemented with additional bread (50 g in total), was homogenized with 110 ml soda and 110 ml of artificial saliva in the presence or absence of AN-PEP (200 mg pure enzyme/g protein) and introduced in the TIM-system.

Sampling and analysis

During the transit of the homogenized food products through the compartments of the TIM-system, samples of 2 ml were taken at time points: 0, 15, 30, 45, 60, 90, 120, 150, 180 and 240 minutes from the stomach, duodenum, jejunum and ileum compartments. The samples were snap frozen in dry ice to stop enzymatic activity.

Before analysis, the samples were thawed and AN-PEP activity was stopped by increasing the pH to 11-12 using 1 M NaOH, followed by neutralization with 1M HCl. Hereafter the samples were kept at 85° C for 10 minutes to inactivate any residual enzymatic activity. Identical volumes from each sample were centrifuged for 10 minutes at 14.000 rpm to separate the water-soluble and water-insoluble components. The water-insoluble fractions were solubelized in the same volume of 6x protein sample buffer (60% glycerol, 300 mM Tris (pH 6.8), 12 mM EDTA pH 8.0, 12 % SDS, 864 mM 2-mercaptoethanol, 0.05% bromophenol blue). From these solutions 2 µl from each sample was used for the protein and Western blot analysis.

Synthetic peptides

Peptides were synthesized as described previously (32).

MAb against the Glia-α20, Glia-α9, Glia-γ1, LMW and HMW glutenin T cell stimulatory epitopes

The specificity and the IgG subclass of the antibodies used in this study are presented in Table 1.

For the generation of a mAb specific for the α-gliadin derived T cell stimulatory epitope Glia-α20, mice were immunized with peptides chemically cross-linked to tetanus toxoid (TTd-DDDXPFRPQQPYPQP-amide). Fusion and screening of the hybridomas was performed as described (32). The minimal epitope of the anti-Glia-α20 mAb (FRPQQPYP) was determined using a set of partially overlapping 17-mer synthetic peptides.

Table 1. Overview of mAbs specific for T cell stimulatory epitopes involved in celiac disease.

Specificity	T- cell epitope	mAb specificity	mAb subclass
α-Gliadin	Glia -a9	Q**LQPFPQPQ**LPY	IgG1 (k light chain)
	Glia-a20	QPQP**FRPQQPYPQ**PQP	IgG1 (k light chain)
γ-Gliadin	Glia-γ1	QPQQPQQSFPQ**QQRPFI**	IgG1 (k light chain)
LMW- glutenin	Glt-156	Q**PPFSQQ**QQSPFSQ* QPPFSQQQ**QSPFS**Q**	IgG3 (k light chain) IgG1 (k light chain)
HMW-glutenin		QQ**GYYPTSP**QQSG	IgG1 (k light chain)

* antibody used for staining western blots

** antibody used in competition assays experiments

Competition assays for the quantitative detection of T cell stimulatory epitopes

Competition assays were performed as described earlier (16;32). Microtitre plates (Nunc Maxisorb, Immunoplate; Nunc, Copenhagen, Denmark) were incubated over-night with 2–5 µg/ml mAb in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.2, at room temperature. Plates were washed in PBS/0.02% Tween-20 and residual binding sites were blocked with PBS/ 1% skim milk powder (Fluka, Zwijndrecht, the Nether-lands). Of the gluten containing samples, different dilutions were made in a buffer containing 50 mM Na_2HPO_4/NaH_2PO_4 pH 7.0, 150 mM NaCl, 0.1% Tween-20/ 0.1% skim milk and a protease inhibitor cocktail (Complete, Roche Diagnostics GmbH, Penzberg, Germany). For the detection of gliadins these were mixed with either a biotinylated Glia-α9 or Glia-γ1 gliadin T cell epitope encoding peptides (32). For the detection of the Glia-α20 T cell stimulatory epitope a biotinylated peptide encoding the Glia-α20 epitope was used as the indicator peptide (Bio-XKAKAKAKAXPFRPQQPYPQP-amide). For quantification, a standard curve was made using the Prolamine working group (PWG) gliadin reference material (33) in the concentration range 10 µg/ml-10 ng/ml mixed with biotinylated indicator peptides. The assays, specific for the detection of T cell stimulatory epitopes of LMW glutenin, were calibrated using a 25-mer synthetic peptide as standard that contains the Glt-156 epitope (16). The HMW-glutenin specific assay was calibrated using a chymotrypsin digest of six purified HMW-glutenin proteins (kindly provided by P. Shewry, Rothamsted Research, Hampenden, United Kingdom) Both standards were used in a concentration range from 1 µg/ml-2 ng/ml.

The mixtures were incubated on plates for 1.5 hours at room temperature. Next, plates were washed and incubated for 30 minutes with streptavidin conjugated horse-radish peroxidase in PBS/ 0.1% skim milk.

Thereafter, bound peroxidase was visualised by incubation with a solution of 3', 3', 5, 5'-tetramethylbenzidine (Sigma-Aldrich Zwijndrecht, the Netherlands). Finally, absorbance at 450 nm was read on a Multiscan plate reader (Wallac, Turku, Finland).

Protein analysis by 1D SDS-PAGE and Western blotting

To determine the level of T cell stimulatory epitopes present in the water-insoluble fractions, these were solubilized in 6x protein sample buffer (60% glycerol, 300 mM Tris (pH 6.8), 12 mM EDTA pH 8.0, 12 % SDS, 864 mM 2-mercaptoethanol, 0.05% bromophenol blue). The samples were separated on a 12.5% SDS-PAGE gels for detection of gliadins and LMW glutenins and on 10% SDS-PAGE gels for detection of HMW glutenins. The proteins were visualized either directly using Imperial Protein Stain (Pierce, Rockford IL, USA), or after transfer to PVDF membranes with the mAbs specific for stimulatory T-cell epitopes from α- and γ-gliadin (32)(and this study) and HMW- and LMW glutenins (16).

T-cell proliferation assay

For the T cell proliferation assays the samples were treated with amylglucosidase and trypsin. Next the samples were incubated O/N at 37°C with guinea pig tissue transglutaminase (200 ug/ml) in the presence of 10 mM CaCl2. Proliferation assays were performed in triplicate in 150 µl Iscove's Dulbecco's medium (BioWhittaker, Vervier Belgium) supplemented with 10% human serum in 96-well flat bottom plates (Costar, Corning Inc., Corning, USA) using 10^4 gluten specific T-cells stimulated with 10^5 irradiated HLA-DQ2-matched allogenic PBMCs (3000 RAD). Of the samples three different amounts of the samples were tested: 0.5 ul, 0.16 ul and 0.05 ul respectively. These amounts were shown to be none toxic for the T-cells. After 48 h incubation at the 37°C, cultures were pulsed with 0.5 µCi of ^{3}H-thymidine, harvested 18h later and the thymidine incorporation was quantified with a liquid scintillation counter (1205 Betaplate Liquid Scintillation Counter; LKB Instruments, Gaithersburg, Maryland, USA).

RESULTS

Digestion of bread and a fast food menu in the TIM-system

To study the effect of AN-PEP on the degradation of gluten proteins in the GI-tract, two experiments were performed. These experiments were designed to represent a small meal (e.g. breakfast) and a more complex meal (e.g. dinner). In the first experiment 70 g of white bread was homogenized together with artificial saliva in the presence or absence of AN-PEP. In the second experiment a quarter of a fast food menu consisting of a sandwich, a hamburger, ketchup, French fries and a glass of soda was homogenized with artificial saliva in the presence or absence of AN-PEP. Since the quantity of bread in this menu was relatively low (16 g), extra bread was added to a total amount of 50 g. After homogenization the mixtures were introduced in the TIM-system, mimicking the human GI-tract (Figure 1). Samples were collected at time points 0, 15, 30, 45, 60, 90, 120, 150, 180 and 240 minutes after the start of the experiment from the stomach, duodenum, jejunum and ileum compartments.

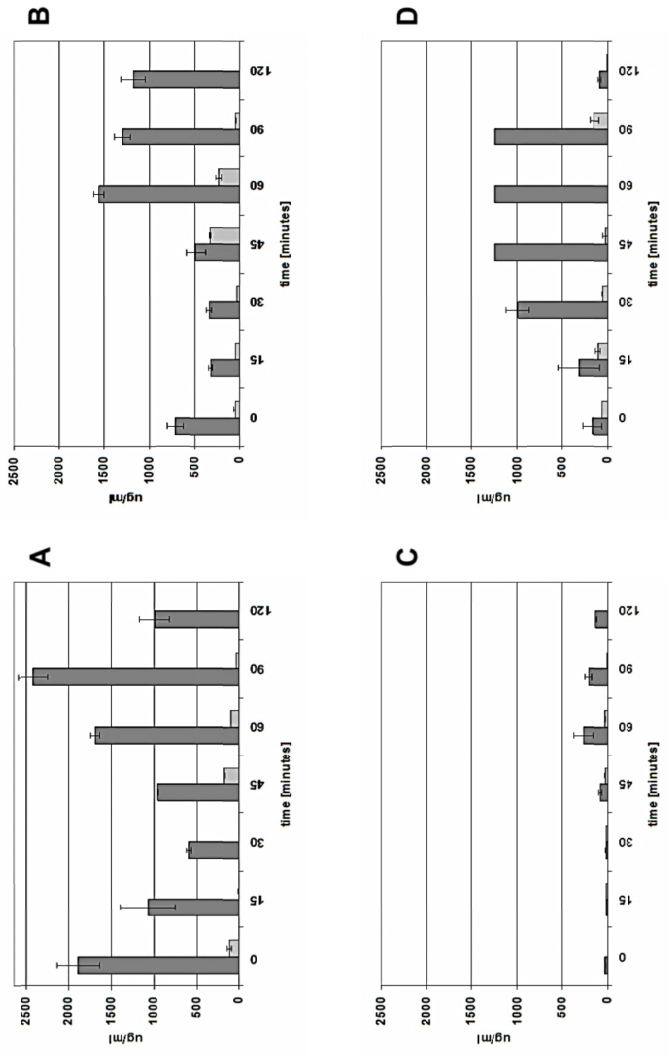

Figure 2. The presence of T cell stimulatory epitopes of α- and γ-gliadin, HMW- and LMW-glutenin in the water-soluble fractions of the white bread digested in the TIM system.

70 g of bread was digested in the TIM system in the presence (gray bars) or absence (black bars) of AN-PEP. At the indicated time points samples were taken from the stomach compartment. The samples were divided in water-soluble and water-insoluble material and the level of T cell stimulatory epitopes in the water-soluble material was determined with mAb based competition assays (16;32)(this study). Levels of the lia-α9 (A), Glia-γ1 (B), HMW-glutenin (C) and LMW-glutenin (D) epitopes in the water-soluble part of stomach fractions.

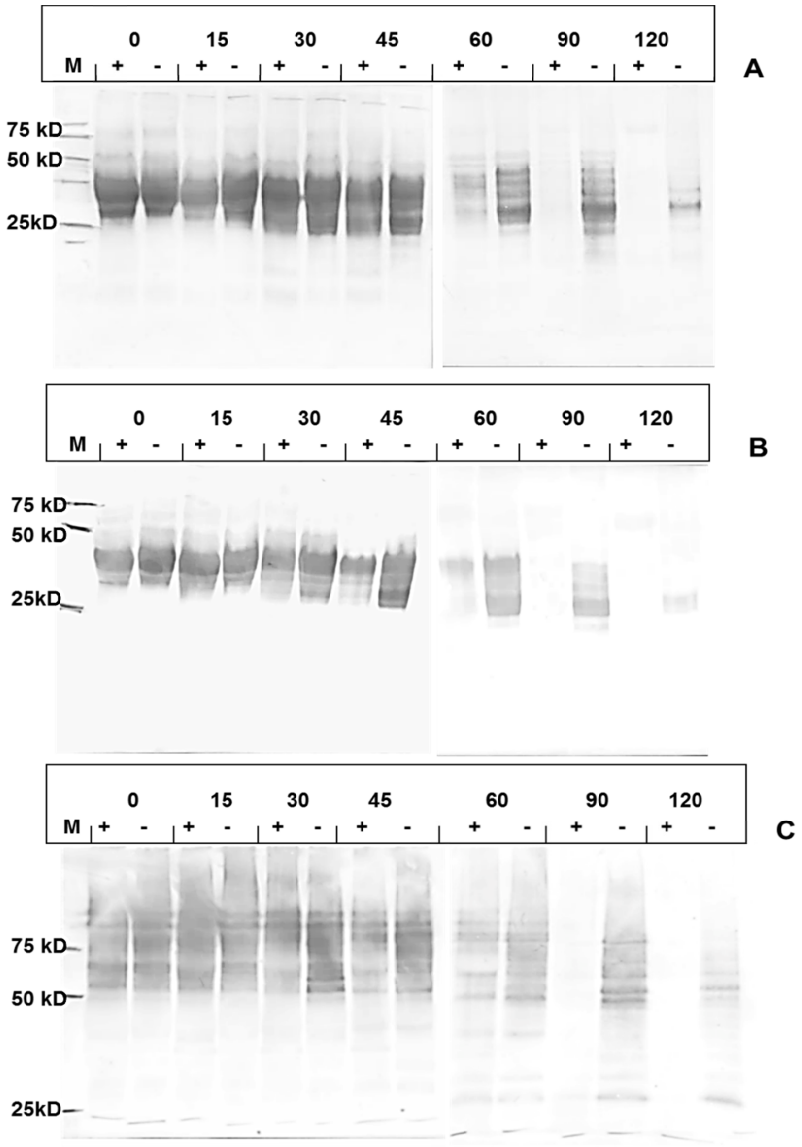

Figure 3. The presence of T cell stimulatory epitopes of α-, γ-gliadin and HMW- glutenin in the water-insoluble fractions of the white bread digested in the TIM system.

70 gram of bread was digested in the TIM system in the presence (+) or absence (-) of AN-PEP. At the indicated time points samples were taken from the stomach compartment and the proteins present in the water-insoluble part of the fraction were separated by 1D SDS-PAGE. After transfer to a PVDF membrane the presence of proteins containing T cell stimulatory epitopes of Glia-α9 (A), Glia-γ1 (B) and HMW-glutenin (C) were visualized using epitope specific mAb (16;32).

In order to study the degradation of gluten in the TIM-experiments, three types of assays were performed. The presence of gluten peptides in the water-soluble fractions was measured by monoclonal antibody (mAb) based competition assays specific for T cell stimulatory peptides present in gliadin (Glia-α9, Glia-α20, Glia-γ1) and LMW- and HMW-glutenin (16;32) (this study). The presence of T cell stimulatory epitopes from gluten in the water-insoluble fractions was determined by Western blot analysis. Moreover samples of the fast food menu taken at 60 minutes after the onset of the experiment were tested by T cell proliferation assays.

AN-PEP accelerates the degradation of gluten present in white bread

After the introduction of the white bread to the TIM-system, the levels of all gluten peptides tested steadily decreased in the water-soluble compartment in the absence of AN-PEP. However, even after 120 minutes significant levels of gluten peptides were still detectable in the stomach compartment (Figure 2). In contrast, when the white bread was introduced together with AN-PEP the digestion of gliadins (Figure 2A and 2B) and glutenins (Figure 2C and 2D) was accelerated at all time points.

Samples that were collected from the duodenum, jejunum and ileum compartments contained very low levels of gluten peptides. In the case of digestion in the presence of AN-PEP no gluten peptides could be detected in these samples at all (results not shown).

Similarly, the Western blot analysis indicated that the degradation of gluten due to the addition of AN-PEP was accelerated. In the water-insoluble fractions of the stomach compartment α-gliadin, γ-gliadin and HMW-glutenin proteins were detectable (Figure 3). Although a significant degradation of gluten was seen in the absence of AN-PEP, both gliadins (Figure 3A and 3B) and glutenins (Figure 3C) were still present after 120 minutes in the stomach compartment. In the presence of AN-PEP, however, faster degradation of both the gliadins (Figure 3A and 3B) and the glutenins (Figure 3C) occurred. This difference in degradation is already apparent after 45 minutes, and after 90 minutes gluten proteins could no longer be detected in the AN-PEP treated fractions (Figure 3).

The duodenum fractions contained only a small amount of water-insoluble material. Western blot analysis failed to detect intact gluten proteins in these fractions, neither in the absence nor presence of AN-PEP (results not shown).

AN-PEP accelerates the degradation of gluten present in a complex meal

In the second experiment the digestion of gluten in a fast food menu was monitored. In the absence of AN-PEP, gluten peptides were found to be degraded in the water-soluble fraction. However, residual gluten peptides were still detectable in the fractions collected from all compartments up to 150 minutes after the start of the experiment (Figure 4). In the presence of AN-PEP, the degradation of gliadins (Figure 4B) and glutenins (Figure 4D) was accelerated. In the absence of AN-PEP, gluten peptides could be detected in the samples that were collected from the duodenum, jejunum and ileum compartments (Figure 4A and 4C). In the presence of AN-PEP much lower amounts of gluten peptides could be detected in these samples (Figure 4B and 4D).

Again, the analysis of the water-insoluble material collected from the stomach and duodenum compartment indicated accelerated degradation of the gluten in the meal. In the presence of AN-PEP the amount of α-gliadin is clearly decreased in the stomach compared to the digestion in the absence of AN-PEP (Figure 5A). Directly after homogenization (t = 0 min) a clear difference is apparent and after 60 minutes no more gluten proteins could be detected in the AN-PEP samples while such proteins were clearly still present in the control samples. Similarly, the HMW-glutenins were degraded faster in the presence of AN-PEP (Figure 5C). After 60 minutes no more HMW-glutenins could be detected in the AN-PEP fractions while partially degraded HMW-glutenins were still present in the control fractions (Figure 5C). Finally, the degradation of the γ-gliadins and LMW-glutenins was similarly enhanced by the addition of AN-PEP (Figure 5B and 5D).

Similar to the white bread experiment, the amount of water-insoluble material in the duodenal fractions was too small to allow detection of gluten proteins (result not shown).

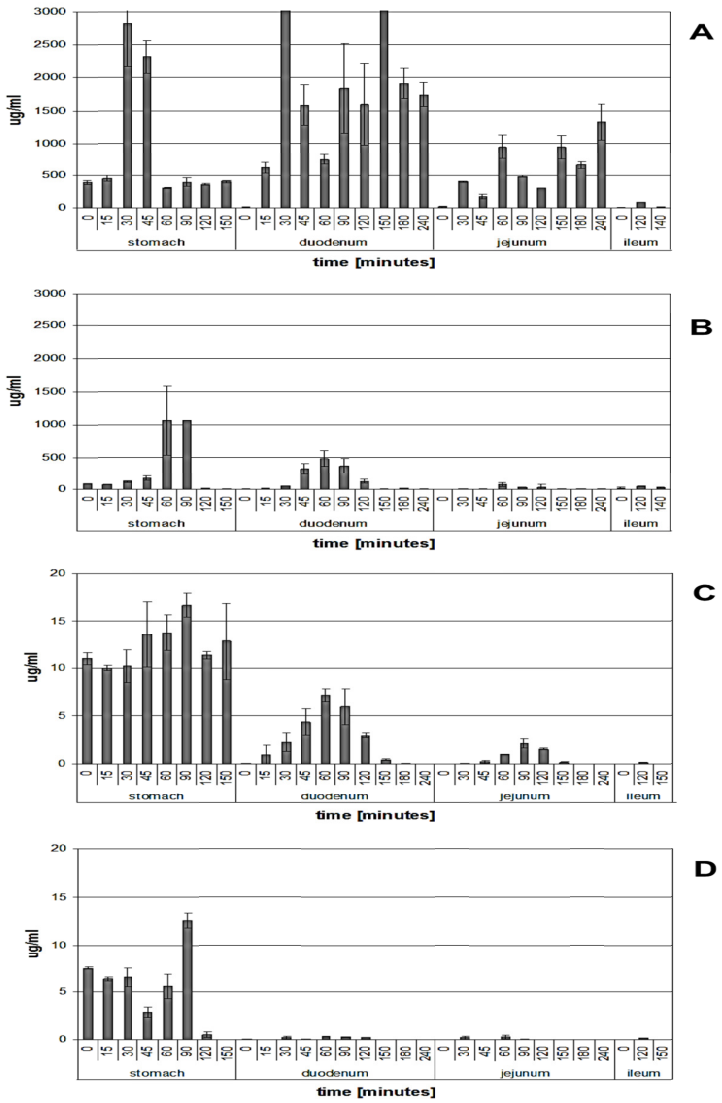

Figure 4. The presence of T cell stimulatory epitopes of α-gliadin and HMW-glutenin in the water-soluble fractions of a fast food menu digested in the TIM system.

A fast food menu, supplemented with additional bread (in total 50 g bread) was digested in the TIM system in the presence or absence of AN-PEP. Samples were taken at the indicated time points from the stomach, duodenum, jejunum and ileum compartments. The samples were divided in water-soluble and water-insoluble material and the level of T cell stimulatory epitopes in the water-soluble material was determined with mAb based competition assays. Levels of the Glia-α20 gliadin epitope (this study) in the absence (A) and presence (B) of AN-PEP. Levels of the HMW glutenin epitope (16) in the absence (C) and presence (D) of AN-PEP.

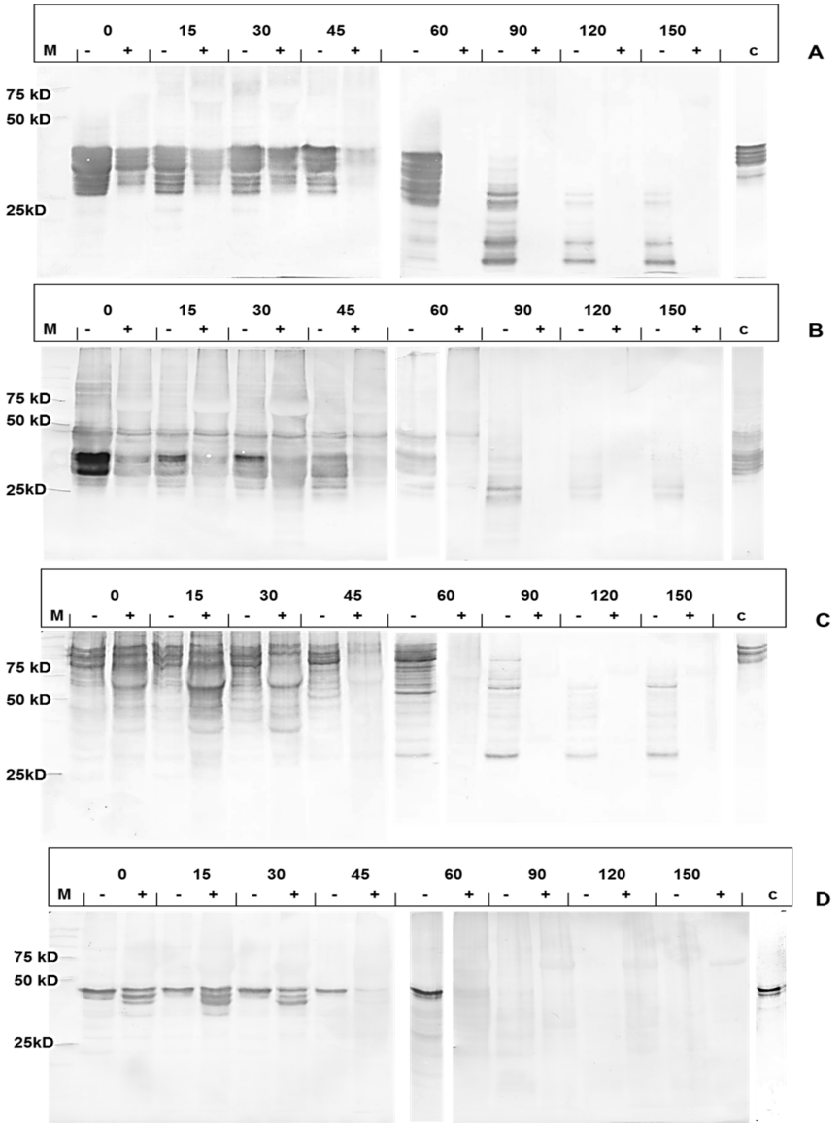

Figure 5. The presence of T cell stimulatory epitopes of α- and γ-gliadin, HMW- and LMW-glutenin in the water-insoluble fractions of a fast food menu digested in the TIM system.

A fast food menu, supplemented with additional bread (in total 50 g of bread) was digested in the TIM system in the presence (+) or absence (-) of AN-PEP. At the indicated time points samples were taken from the stomach compartment and the proteins present in the water-insoluble part of the samples were separated by 1D SDS-PAGE. After transfer to a PVDF membrane the presence of proteins containing T cell stimulatory epitopes of Glia-α20 (A), Glia-γ1 (B), HMW-glutenin (C) and LMW- glutenin (D) were visualized using epitope specific mAb (16;32) (this study). In this figure c is a gluten control.

Small amounts of gluten peptides could still be detected in the AN-PEP treated samples that were measured with the antibody based competition assays. As these assays detect gluten fragments that are smaller than those required for T cell stimulation, we determined if AN-PEP destroyed the T cell stimulatory properties in the TIM-system. T cell clones specific for the Glia-α9, Glia-α20, LMW glutenin and the HLA-DQ8 restricted HMW glutenin derived T cell epitopes were incubated with the untreated and the AN-PEP treated samples and T cell proliferation was measured (Figure 6). While in the untreated samples T cells stimulatory activity was clearly present, AN-PEP treatment completely abolished this activity.

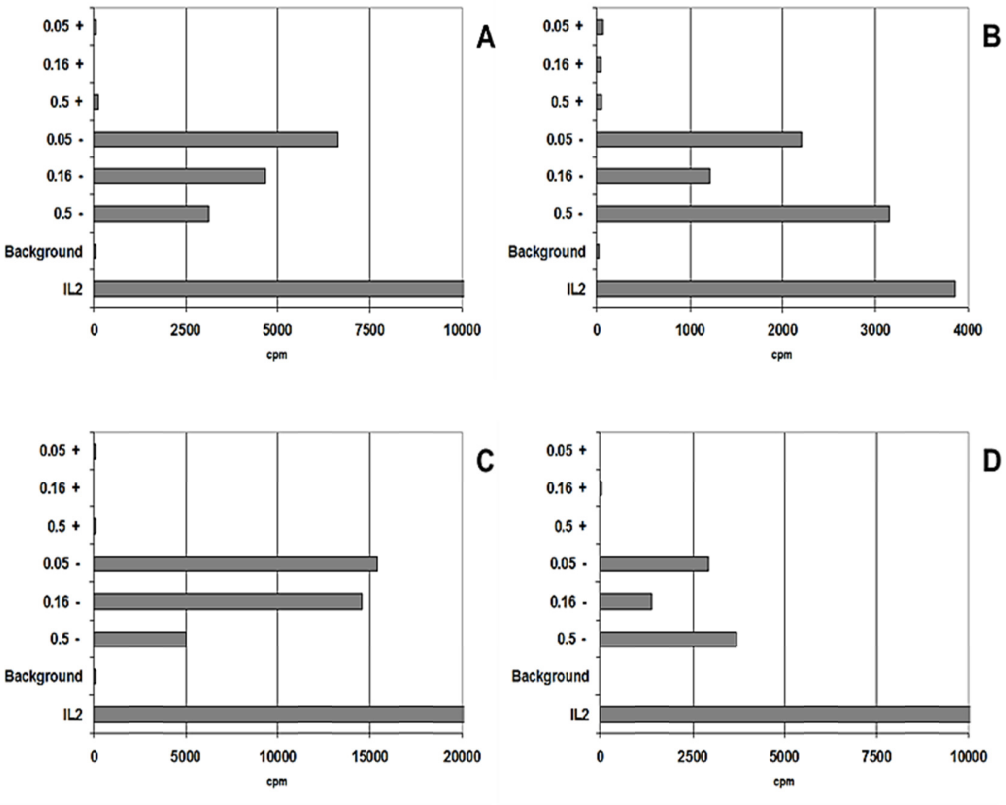

Figure 6. Stimulation of gluten specific T cells by samples of the fast food menu collected at 60 minutes after the addition of AN-PEP from the stomach compartment of the TIM system.

Three different concentrations of the stomach samples: 0.5 µl, 0.16 µl and 0.05 respectively collected from the experiment of the fast food digestion in the presence (+) or absence (-) of AN-PEP were tested in T cell proliferation assays. Stimulation of T cell clones recognizing the Glia-α9 epitope (A), Glia-α20 epitope (B), the HLA DQ8 molecule restricted HMW-Glt epitope (C) and LMW Glt-156 epitope (D) is shown.

DISCUSSION

It is well established that CD patients are intolerant to gluten and that a strict gluten-free diet (GFD) is an effective treatment for CD. The lifelong maintenance of such a diet, however, is hard to achieve. First of all, the diet in the western world is heavily based on gluten containing foods like bread and pasta. In addition, because of its special properties, like its elasticity and capacity to bind water, gluten is often added to foods that would otherwise be gluten-free. Finally, many foods that are rendered gluten-free may still contain traces of gluten while naturally gluten-free ingredients are often contaminated with gluten. CD patients, therefore, can inadvertently be exposed to gluten. Moreover, a GFD can cause social constraints and this can lead to non-compliance with the diet. For these reasons an alternative to a GFD would be useful to patients. Oral supplementation with enzymes to degrade gluten before it causes damage has been suggested for this purpose. Initially this proposition was based on the theory that CD is caused by an enzyme deficiency (34) and the use of non-human proteases for gluten detoxification was already proposed in the late fifties (35). Recently a clinical trial was performed in which an extract from animal intestines was used. It was shown that the enzyme therapy offered better protection than placebo. However, the differences between enzyme therapy and placebo were small and no complete protection was given by the extract. Furthermore, not all patients did benefit to the same extent from the enzyme supplementation (36).

As gluten is rich in the amino acid proline, other recent studies have used post-proline cutting enzymes. Promising enzymes tested are the prolyl oligopeptidases from *Flavobacterium meningosepticum, Sphingomonas capsulate*, and *Myxococcus xanthus*. These enzymes are capable of degrading proline containing peptides that are otherwise resistant to degradation by proteases in the gastrointestinal tract *in vitro* (23;37;38). However, the suitability of these enzymes to degrade gluten *in vivo* is questionable since the enzymes have a pH optimum between 7 and 8 and do not function at the acid pH of the stomach. Moreover, they are efficiently broken down by pepsin (23). Finally, due to their structure, in which a β-propeller domain restricts entry into the active site of the enzymes, they preferentially cleave short peptides (39). Encapsulation of these prolyl oligopeptidases was proposed to protect them against gastric juice (38). However, in a recent *ex vivo* study, using biopsy-derived intestinal tissue mounted in Ussing chambers, it was observed that only high dosages of prolyl oligopeptidase tested were capable to eliminate the accumulation of immunogenic peptides in the serosal compartment (21). This indicates that, even if the enzyme is encapsulated, due to the relatively low efficiency the prolyl oligopeptidase will not be able to degrade gluten before it reaches the proximal part of the duodenum, the site where gluten triggers inflammatory T cell responses. To overcome these problems a combination therapy has been proposed. For this the prolyl oligopeptidase is combined with a cysteine endoprotease from barley whose natural function is to degrade the gluten-like molecules in barley, to the benefit of the germinating seed. While such a combina-

tion therapy may prove effective, a therapy based on one enzyme would have obvious advantages (40).

Recently, we have investigated a prolyl endoprotease of *Aspergillus niger,* AN-PEP (31). We observed that AN-PEP has clear advantages over prolyl oligopeptidases as it is much more efficient in degrading gluten peptides, is active at low pH, and resistant to pepsin degradation (31;41). Moreover, as AN-PEP is a prolyl endoprotease, it degrades gluten peptides as well as intact gluten proteins (31;41).

These results indicated that AN-PEP may be suitable as an oral supplement for gluten degradation but its efficacy *in vivo* remained to be established. Although this can be tested in animal models, as has been carried out for a prolyl oligopeptidase, the relevance of this for the human situation is debatable as the gastrointestinal tract of animals only partly reflects that of humans. We have therefore chosen to study the efficacy of AN-PEP using a dynamic gastrointestinal model that closely mimics the *in vivo* conditions found in the human stomach and small intestine (26-28). Moreover, we have tested the capacity of AN-PEP to degrade gluten when present in a relatively simple food matrix, e.g. white bread, as well as when gluten is present in complex food matrix, e.g. a complete fast food meal. The results of this study show that AN-PEP is indeed capable of degrading gluten under conditions found in the human GI-tract. We have carried out three types of analysis: competition assays that measure the presence of small gluten fragments in the water-soluble fractions, Western blot to measure intact gluten proteins and relatively large fragment thereof (> 5 kD) and T cell proliferation experiments that measure the presence of gluten derived T cell stimulatory peptides. Both the competition assays and the Western blot analysis gave comparable results: the disappearance of gluten proteins as indicated by Western blot analysis also led to a disappearance of smaller gluten peptides as measured in the competition assay. Moreover T cell proliferation assays on samples collected at 60 minutes from the digesting fast food menu showed that after the addition of the AN-PEP, the stomach content is no longer capable to induce a T cell proliferation. Taken together, these results indicate that AN-PEP completely degrades gluten into harmless fragments within 2 hours, the average passage time of food in the stomach. Besides, during the stay of the gluten in the stomach, the level of T cell stimulatory epitopes of α- and γ-gliadin HMW-glutenin and LMW-glutenin epitopes in the water soluble fragments were significantly lower in the presence of AN-PEP than in the absence. This results in much lower amounts of T cell stimulatory epitopes that are released from the stomach into the duodenum, thereby reducing the exposure of the proximal duodenum with T cell activating peptides. Moreover, in a previous study we have already demonstrated that AN-PEP will efficiently cut the alpha-gliadin peptide 31-43 and thus destroy the innate stimulatory properties of gluten.

It is important to note that our results may underestimate the rate of gluten degradation in the human body. Although the TIM-system simulates the lumen of the human GI-tract, including peristaltic movements, secretion of juices from the salivary gland, the stomach, pancreas and liver, no brush border enzymes are present in the model. As brush border enzymes have an additive effect on proteolysis, which is also shown for the breakdown of gluten proteins (25), it is likely that the degradation of gluten is even more efficient than indicated by our results.

In conclusion, we have tested the effect of the addition of AN-PEP on gluten degradation in a system that closely mimics the conditions in the upper human GI-tract. Our results demonstrate that within the time span that food is normally present in the stomach, co-administration of AN-PEP led to a complete disappearance of T cell stimulatory peptides of gliadins and glutenins. Importantly, our results demonstrate that AN-PEP is capable of degrading gluten when this is present in a complex food matrix. Moreover, AN-PEP is derived from the food grade microorganism *Aspergillus niger* and available on industrial scale. Ultimately, clinical trials will be required to determine if oral enzyme supplementation can remove all gluten toxicity. Our results indicate that AN-PEP is a very suitable candidate for testing in such trials.

FUNDING

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STATEMENT

COMPETING INTERESTS

L. Edens is an employee of the DSM Food Specialties. Part of the work presented here has been patented by DSM.

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Chapter 6

Discussion

DISCUSSION

Currently the only treatment for celiac disease is a lifelong gluten-free diet. While very effective it is cumbersome as wheat based products are so commonly used in our daily diet. Many food products are therefore off limits for celiac disease patients. Moreover, gluten is often present as a hidden ingredient which can lead to inadvertent exposure to gluten. While many patients are content with their gluten-free diet, others would welcome alternative treatments and/or food products that would allow more flexibility. The research presented in the present thesis focuses on the development of such alternatives.

Celiac disease is an HLA-associated disease: it develops almost without exception in HLA-DQ2 and/or HLA-DQ8 individuals (1-3). There are many other HLA-associated diseases, like type I diabetes and rheumatoid arthritis, but only in celiac disease the disease-causing agent is known: wheat-derived gluten and similar proteins in other cereals. Around 1950 Willem Karel Dicke first observed the connection between cereal intake and the presence of symptoms in children with celiac disease (4). He found that during the Second World War, when wheat products were scarce, his patients got better but relapsed when wheat was reintroduced after the war. Later research demonstrated that the symptoms were caused by the gluten and gluten-like storage proteins in wheat and related cereals (5). Based on these observations the gluten-free diet was introduced: when a patient does not consume wheat, barley and rye products she/he does not experience any symptoms. The definition of a treatment is that it treats a problem and may lead to its cure but more often the treatment ameliorates a problem only for as long as it is applied. This is also the case in celiac disease, the gluten-free diet offers release of symptoms only as long as it is followed and therefore must be maintained lifelong. It's main disadvantage is that it can restrict participation in social events, traveling and eating out which causes more problems to the newly diagnosed and to young patients.

In the present thesis three alternative approaches to the gluten-free diet are presented and discussed. These approaches are: the development of cereals or gluten with no or low immunogenic content, the use of oats as an alternative cereal, and the use of enzymes for gluten detoxification.

Better detection methods

In order to determine gluten toxicity, appropriate reagents need to be available to detect such toxicity. Earlier studies had defined the epitopes responsible for the immune response. These were found to be peptides derived from both gliadins and glutenins (6-9). All such peptides have been identified with the use of patient derived T cells specific for such peptides when bound to the disease predisposing HLA-DQ2 or HLA-DQ8 molecules. Therefore, the golden standard for testing the immunogenicity of food products would be the use of such gluten specific T cells isolated from the small intestine of celiac patients. Unfortunately, they can not be used for routine screening purposes as they are highly sensitive for various compounds commonly found in food,

can only be employed in a sterile environment and testing is time consuming and expensive. For this reason antibody-based systems are commonly employed. The validated gluten detection method currently employed is based on the R5 monoclonal antibody that was raised against a barley epitope and also reacts with sequences from gliadins and rye (10,11). Unfortunately, the epitope recognized by the R5 antibody does not represent the known toxic sequences of gluten and therefore does not measure gluten toxicity. Ideally, antibody-based gluten detection systems would employ antibodies that are raised against known T cell stimulatory sequences in gluten and have a reactivity pattern that closely resembles that of gluten specific T cells.

For this purpose five monoclonal antibodies were raised in our laboratory that detect known gluten epitopes namely: Glia-alpha-20, Glia-alpha-9, Glia-gamma-1, the LMW-glutenin 156 and the HMW glutenin epitope (12-14). These antibodies were used to set up a competition based assay that can detect the presence of these epitopes both in peptides mixtures and proteins. All 5 antibodies were shown to be suitable for gluten detection in Western blot analysis as well. The fine specificity of these antibodies for gluten and gluten-like peptides and proteins was determined and compared with that of the gluten specific T cells (Chapter 2). It was shown that, just like the gluten specific T cells the antibodies react with homologous peptides found in epitopes from barley, rye and triticale and that the specificity partially overlaps with that of the corresponding T cells (15). Such antibody reagents are therefore very suitable for the detection of gluten, in particular sequences that are linked to gluten toxicity for celiac disease patients. Therefore these antibodies were used in the subsequent studies aimed at the development of alternatives for the gluten-free diet.

Moreover, in collaboration with the diagnostics company Europroxima, one of the antibodies has been used to generate a gluten detection kit that has been tested in an international RING-trial in 2009/2010 in which 10 laboratories participated. As the results of this trial were positive and the new kit has several advantages over the commercially available R5 test kit, it was launched on the market in June 2010 and may ultimately replace the R5 based method.

Towards low toxic wheat and safe gluten proteins

As wheat is so important for food production in industrialized countries, one would like to have a wheat variety available that would be suitable for consumption by celiac disease patients. With the identification of the immune stimulatory sequences in the gliadins and glutenins it became possible to investigate if differences exist between wheat varieties or gluten proteins with regard to toxicity for celiac disease patients. Indeed, it could be shown that natural variation exists in the immunogenic content of wheat varieties, especially between varieties belonging to different genotypes (14,16). This sparked the hope that a non-toxic hexaploid bread wheat variety could be identified. The benefits of a non-toxic hexaploid wheat are obvious as they have good crop yield and good baking quality and could thus be easily used in the food industry. Ideally, this would form the basis for the production of food products that would have the same nutritional value and the smell, texture and taste typical for normal wheat based products. Further research, however, indicated that it is highly unlikely that natural wheat varieties exist that are safe for consumption by patients. The analysis of over

3000 gliadin genes indicated that none encoded a protein that would be entirely devoid of toxicity. Moreover, all ancient wheat varieties that we tested proved to contain immunogenic epitopes, although to a lesser extent than the hexaploids bread wheat varieties.

The results, however, offered an alternative approach to eliminate gluten toxicity (Chapter 3). Extensive testing of all existing variants of known immunogenic peptides in gluten proteins indicated that some of these variants lacked immunogenic properties. For example, a variant peptide present in the A genome contains one single amino acid substitution that rendered the epitope non-immunogenic. This single amino acid substitution concerned the replacement of the proline at relative position 8 in the peptide by a serine. Interestingly the majority of HLA-DQ2-restricted gluten epitopes contain a proline at this position (17) moreover, we observed that the introduction of this proline to serine substitution eliminated the immunogenic properties of all these peptides, including the highly immunogenic 33-mer found in the alpha-gliadin proteins. We used this knowledge to device a general strategy to eliminate gluten toxicity in alpha-gliadin proteins and provided proof of principle at the peptide level. Similar approaches are likely to be applicable to other gluten proteins as well. These results can now be applied and may lead to large scale production of safe gluten proteins for incorporation in “gluten-free” foods meant for celiac disease patients.

Thus, in our search for a non-toxic cereal we failed but we did find a way to detoxify gluten.

Alternative cereals: oat

While it is well established that wheat, barley and rye can not be tolerated by celiac disease patients, oat is considered a reasonable safe alternative. In the last 10 years, however, the introduction of oat in the gluten-free diet was surrounded by controversy since not all patients tolerate oat (18). “Oat toxicity” was partially explained by the discovery that the large majority of oat samples were contaminated with wheat, barley or rye during cultivation, transport and/or processing (19). To overcome this problem contamination-free oat chains have been set up in Scandanavia and more recently also in The Netherlands. Another problem surrounding oat is that it was found that gluten reactive T cells can cross react with peptides derived from the gluten-like avenin proteins in oat and the isolation of avenin specific T cell clones from a celiac disease patient that was intolerant to oat (7,20). Thus, oat does contain some peptides that can elicit immune responses but the level of exposure is apparently so low that most patients can consume oat without problems. To determine if all oat varieties are equally immunogenic we tested a representative collection of oat samples used in the Netherlands (Chapter 4). We observed that all oat samples tested contain immunogenic epitopes but that their presence is variable. Thus, just like in wheat, also in oat there is a natural variation in the immunogenic content. We also identified a monoclonal antibody which specifically detects avenin epitopes, the use of which will make the testing for intrinsic toxicity easier. The results indicate that though testing of the immunogenicity of oat, low or non-toxic varieties may be identified that will allow oat consumption by all celiac patients without any constraint.

The use of enzyme therapy for gluten detoxification

Oral supplementation with enzymes in order to diminish the exposure of patients to hidden gluten was proposed already in the late 1950 when was thought that celiac disease was due to a lack of enzymes (21,22). The first enzyme of choice was papain, but the results of the experiments were poor (22). The following attempt was performed with an extract from animal intestine (23). This clinical trial did show a certain protection in comparison to the placebo, but not all patients benefited to the same extent. Since the high proline content of gluten is one of the reasons for its poor degradation in the gastro-intestinal tract the use of post-proline cutting enzymes was proposed (24-26). Promising enzymes that were tested are the prolyl oligopeptidases from Flavobacterium meningosepticum, Sphingomonas capsulate and Myxococcus xantus, which were shown to cut proline rich gluten proteins (25-28). These enzymes, however, have a drawback since they can not function in the acid milieu in the stomach and can also be degraded by pepsin. They are thus not able to degrade gluten before it reaches the upper part of the small bowel where the characteristic celiac disease lesions are found.

An alternative enzyme is the prolyl endoprotease of *Aspergillus niger*, AN-PEP. It was already shown that this enzyme has several advantages over the previously described enzymes since it is more efficient in degrading gluten peptides, is active at a low pH and is resistant to pepsin degradation (29). During my research we took the testing of this enzyme to the next level and we showed that AN-PEP is capable to degrade gluten present in a simple bread meal and also in a more complex bread containing meal in a system that mimics the human gastro-intestinal tract (Chapter 5). The samples recovered from the “digested” meal were found to no longer contain immunogenic sequences when tested with specific monoclonal antibodies and more significantly with gluten specific T cells. These promising results form the pre clinical phase of a clinical trial which has already shown that the enzyme can be safely consumed by patients (Table 1). A larger clinical trial will have to demonstrate that the enzyme is useful for gluten degradation in humans and can prevent the reappearance of symptoms when patients consume gluten.

Table 1. Description of AN-PEP clinical trial

Phase	Description	Results
pre clinical	*In vitro* experiments	Effective degradation of known epitopes in food products
I	safety, pharmacokinetics, administration way	no side effects by oral administration
II	determination of optimal dose	not performed
III	comparison to known therapies, testing combination therapies and different doses	not performed
IV	long term efficacy and effects	not performed

The future: from bench to the patient

The best reward for any research is to see it applied. The focus of my research has been on the development of alternatives to the current gluten-free diet. Parts of the results presented in this thesis are closer to application than others. The detection of immunogenic gluten and gluten-like epitopes with the use of monoclonal antibodies is closest to being exploited and the anti-Glia-alpha-20 antibody is already incorporated in a detection kit which has been successfully tested in a ring trial. Since this kit detects a non-repetitive immunogenic gluten epitope and uses a synthetic peptide as a standard, it offers a better gluten quantification method compared to the existing method based on the R5 antibody. The kit was launched on the market in June 2010. While this kit is primarily meant for industrial use, it may be possible to design a variant that would allow patients to test food products themselves.

Also, the results on oat toxicity can be readily applied to ensure safety of oat consumption by patients. Unfortunately, the situation is much more complex when it comes to wheat. Large scale screening of gliadin genes indicated that non-toxic gliadin proteins do not exist. Accordingly, all tested hexaploid, tetraploid and diploid wheat varieties were found to contain toxic epitopes. It is thus highly unlikely that true non-toxic wheat can be obtained through conventional breeding programs. However, during these screening programs we discovered naturally occurring amino acid mutations in gliadin epitopes which eliminate the T cell stimulatory properties of these epitopes. This now allows the design and expression of modified gliadin genes for the production of non-toxic gluten. As the introduced amino acid modifications are naturally occurring it is expected that the modified proteins will have retained much of the desired technological properties and nutritional value of the original gluten proteins. Likewise, a similar detoxification method could be used to eliminate the toxicity of the gamma–gliadins, LMW- and HMW-glutenins, allowing the production of complex but non-toxic mixtures of gluten proteins. Although this approach is straightforward, the realization of “Safe Gluten” is still not around the corner. The genetic code of gluten genes will have to be changed and expressed in recombinant fashion, creating genetically modified crops producing recombinant gluten proteins. Apart from public acceptance and regulatory issues, it is unknown how efficient the production would be and it remains to be demonstrated that such “Safe Gluten” is indeed safe for consumption by patients. It will be several years before this point is reached.

Gluten with low dose epitope content can be obtained on a much shorter term either through selection of varieties with low epitope content of by specific amino acid mutations at key positions in known epitopes. By replacing gluten with this “lower epitope dose” gluten the risk of development of celiac disease may be lowered resulting is a reduced incidence of the disease.

The introduction of enzymes for gluten detoxification may be much closer at hand. After demonstrating that AN-PEP can degrade gluten in an artificial gastrointestinal system, the enzyme was tested in a small group of patients which showed that its consumption is not associated with adverse symptoms. Next it will have to be tested if AN-PEP can offer patients good protection against immunogenic wheat present in the diet. For this a large scale clinical trial needs to be executed. At present it is not clear if

the enzyme can offer protection against all wheat containing products since it needs to have access to the gluten in the food matrix and it is likely that this differs between food products. Therefore, it is unpredictable if AN-PEP will ever be able to replace the gluten-free diet. However, considering that AN-PEP has excellent gluten degrading capacity *in vitro*, it is likely that AN-PEP can be applied to safeguard celiac disease patients against unwanted gluten intake, for example during traveling or eating out.

Future prospects

While Dicke recognized already around 1950 that wheat and related cereals were causing celiac disease, it was not until the end of the last century that the molecular basis for this was unraveled. Now we know that gluten contains a multitude of immunogenic peptides that can be modified by tissue transglutaminase and bind to the disease predisposing HLA-DQ2 or -DQ8 molecules and trigger inflammatory T cell responses. Although much is still unknown – i.e. why do not all HLA-predisposed individuals develop disease and what triggers inflammatory responses in the epithelium? – we can try to exploit the current knowledge to the benefit of patients. This was the goal of the work described in the present thesis. In particular it was investigated if it would be possible to develop wheat or gluten proteins that would be safe for consumption by patients and if it is possible to enhance gluten degradation with a post-proline cutting enzyme isolated from *Aspergillus niger*. With the development of safe wheat patients would be able to consume a "normal" diet. Moreover, such wheat could be used to prevent the development of celiac disease in children born in high risk families. On the other hand, co-administration of a gluten degrading enzyme with a gluten-containing meal might allow patients to consume such meals. These two approaches are thus seemingly each others enemy: safe wheat would eliminate the need for enzymatic degradation while an effective enzyme therapy would eliminate the need for safe wheat. In reality the truth lies in the middle. There is no safe wheat and the development of safe gluten is still several years away from actual realization. It is also largely unknown how cost effective such safe gluten proteins can be produced and incorporated in foods for patients. On the other side it is unclear if enzymatic degradation of gluten in the gastrointestinal tract will be so effective that it will allow the introduction of a normal gluten containing diet. Thus, both options are still open and deserve to be explored further.

Next to better treatment options for patients, prevention of celiac disease development is an ultimate goal of research in this area. Given the importance of cereal-based products for nutrition worldwide exposure to gluten is unlikely to change significantly in the future. Reduction of the incidence of celiac disease can thus only be achieved by targeted intervention in high risk families. Delayed introduction of gluten and deliberate exposure to low amounts of gluten early in life are two approaches that are currently being tested. Ultimately, better markers for prediction of the risk of disease development will be required for the design and implementation of more specific intervention protocols. While genetic studies are rapidly identifying such markers, these studies also demonstrate that many gene variants are implicated, each with a low impact. The unraveling of this complex genetic picture will take many years, years in which a gluten-free diet will likely remain the safest option for celiac disease pa-

tients. Nevertheless, it is reasonable to assume that the work described in this thesis will in the near future contribute to the development of novel approaches in celiac disease management.

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Summary

SUMMARY

What is known about celiac disease?

Celiac disease is one of the most common food intolerances, approximately 1% of the population being a celiac disease patient. It is now known that celiac disease is precipitated by ingestion of gluten, the major storage proteins in wheat, and similar proteins in related cereals like barley, rye and triticale (hybrid between wheat and rye). The most common complains of patients consuming gluten are abdominal pain, diarrhea and vomiting. Also neurological symptoms, infertility and retardation of growth can occur. For a positive diagnosis of celiac disease a histological examination of a small bowel biopsy and a clinical improvement upon the introduction of a gluten-free diet is required. None invasive, serological assays are available that measure the presence and titer of IgA antibodies specific for gliadin, deamidated gliadin, endomysium and tissue transglutaminase. Almost without exception celiac disease develops only in genetically predisposed individuals: over 98% of the patients express either HLA-DQ2 or HLA-DQ8. Next to the genetic component also other factors especially environmental play a role in disease development.

Celiac disease is an immune mediated disease, in which gluten peptides come in contact at the level of the small intestine with gluten specific T cells. These T cells could only be isolated from the small intestine of patients and not of healthy controls. Specific gluten sequences rich in prolamine and glutamine amino acids, resist degradation in the gastro-intestinal tract and after deamidation by the enzyme tTg are recognized by T cells. As a result of the inflammation, the mucosa loses its villi and strongly diminishes the absorption surface causing specific symptoms associated with the disease.

The current treatment for celiac disease is strict adherence to a life-long gluten-free diet. The wide-spread use of gluten and gluten-derived starch in the food industry makes the gluten-free diet challenging. It is not surprising that a considerable proportion of patients, especially adolescents, are interested in alternative treatments that would allow gluten consumption.

Thesis content

My project, which makes the content of this book, has focused on the development of alternatives to the gluten-free diet. Two different approaches were investigated: the use of enzymatic supplementation and the identification and/or development of a less/non-toxic cereal.

Chapter 1 is a general introduction to celiac disease.

In **chapter 2** the characterization of monoclonal antibodies raised against T cell stimulatory gluten peptides is described. Their reactivity against the prolamins from wheat, barley, rye and oats was determined and compared with that of gluten reactive T cells. The results demonstrate that the antibody and T cell reactivity patterns overlap significantly, indicating that the antibodies can be used to detect toxic sequences in

gluten. Subsequently, these antibodies were used in the studies aimed at the development of alternative to the gluten-free diet.

In **chapter 3** we propose a new strategy to generate non-toxic gluten. Our experiments demonstrated that non-immunogenic epitope variants were present in certain diploid wheat varieties that differ one amino acid with the toxic variant. Moreover, we found that by the introduction of this naturally occurring amino acid substitution in other toxic epitopes their T cell stimulatory activity was likewise eliminated. This approach can thus be used to generate gluten genes that are devoid of any T cell stimulatory activity and presumably safe for consumption by celiac disease patients.

In **chapter 4** we investigate the safety of oats for consumption by celiac disease patients. We confirmed that commercially available oats are without exception contaminated with other cereals. Perhaps more importantly, we demonstrate that variability exists in the level of T cell stimulatory gluten like peptides in a panel of oats varieties tested, opening the way to select and/or breed oats varieties that contain no harmful gluten-like proteins.

In **chapter 5** we investigate the potential of AN-PEP, a prolyl-endoprotease produced by the microorganism *Aspergillus niger*, to degrade gluten in an artificial gastrointestinal tract system. The enzyme proved very efficient in degrading all toxic epitopes in this system, even when a complex meal was introduced. These “in vitro” studies now justify a clinical trial to assess the safety and effectiveness of the enzyme for gluten degradation in patients.

In **chapter 6** I discuss how the results may lead to novel treatment modalities and novel foods in the near future.

Samenvatting

SAMENVATTING

Wat is bekend over coeliakie?

Coeliakie is een van de meest voorkomende voedselintoleranties, ongeveer 1% van de bevolking is een coeliakie patiënt. Het is bekend dat coeliakie wordt veroorzaakt door inname van gluten, eiwitten in tarwe, en gelijkaardige eiwitten in granen zoals gerst, rogge en triticale (een kruising tussen tarwe en rogge). De meest voorkomende klachten van patiënten zijn buikpijn, diarree en braken. Ook neurologische symptomen, onvruchtbaarheid en vertraging van de groei kunnen optreden. Voor een positieve diagnose van coeliakie is een histologisch onderzoek van een dunne darm biopsie en een klinische verbetering op een glutenvrij dieet nodig. Niet invasieve, serologische testen zijn sinds enkele jaren beschikbaar. Deze meten de aanwezigheid en titer van IgA en/of IgG antilichamen tegen gliadine, gedeamideerd gliadine, endomysium en tTg. Bijna zonder uitzondering ontwikkelt coeliakie zich bij genetisch gepredisponeerde individuen: meer dan 98% van de patiënten zijn HLA-DQ2 of HLA-DQ8 positief. Naast de genetische component beïnvloeden ook andere factoren de kans op ziekte ontwikkeling, zoals infecties en borstvoeding.

Coeliakie is een immuun gemedieerde ziekte waarbij gluten peptiden in contact komen met gluten-specifieke T-cellen in de dunne darm die een ontsteking veroorzaken. Deze T-cellen kunnen alleen worden geïsoleerd uit de dunne darm van patiënten en niet van gezonde controles. Gluten bevat veel sequenties die rijk zijn in het aminozuur proline en daardoor ongevoelig zijn voor degradatie in het maag-darmkanaal. Bovendien bevat gluten het aminozuur glutamine dat door het enzym weefsteltransglutaminase gemodificeerd kan worden. Door deze modificatie ontstaan gluten peptiden die veel beter door T-cellen herkend worden en de ontsteking verergeren. Als gevolg van de ontsteking verliest het slijmvlies van de dunne darm zijn villi waardoor het absorptieoppervlak van de darm sterk vermindert. Dit draagt bij aan de specifieke symptomen.

De huidige behandeling van coeliakie is een strikt levenslang glutenvrij dieet. Het wijdverspreide gebruik van gluten en gluten-afgeleid zetmeel in de voedingsindustrie maakt het glutenvrije dieet uitdagend. Het is niet verwonderlijk dat een aanzienlijk deel van de patiënten, vooral jongeren, geïnteresseerd is in alternatieve behandelingen die gluten consumptie mogelijk zou maken.

De inhoud van het proefschrift

Mijn project, beschreven in dit proefschrift, was gericht op de ontwikkeling van alternatieven voor het glutenvrije dieet. Twee verschillende benaderingen werden onderzocht: het gebruik van enzymatische suppletie en de identificatie en/of ontwikkeling van een minder/niet-toxisch graan.

Hoofdstuk 1 is een algemene inleiding op coeliakie.

In **hoofdstuk 2** wordt de karakterisering van monoklonale antilichamen tegen T-cel stimulerende gluten peptiden beschreven. Hun reactiviteit tegen gluten en gluten-

achtige eiwitten in tarwe, gerst, rogge en haver werd bepaald en vergeleken met die van gluten reactieve T-cellen. De resultaten tonen aan dat de antilichaam en T-cel reactiviteit patronen aanzienlijk overlappen, wat aangeeft dat deze antilichamen gebruikt kunnen worden om toxische sequenties in gluten te detecteren. Vervolgens werden deze antilichamen gebruikt in de studies die gericht waren op de ontwikkeling van alternativen voor het glutenvrije dieet.

In **hoofdstuk 3** stellen we een nieuwe strategie voor om niet-toxische gluten te genereren. Onze experimenten toonden aan dat in bepaalde diploïde tarwe rassen een niet-immunogene gluten variant aanwezig is die één aminozuur verschilt met de immunogenische variant. Bovendien vonden we dat door de invoering van deze natuurlijk voorkomende aminozuur substitutie in andere gluten epitopen hun T-cel stimulerende activiteit eveneens werd geëlimineerd. Deze benadering kan dus worden gebruikt om gluten eiwitten te genereren die geen T-cel stimulerende activiteit meer bevatten en dus vermoedelijk veilig zijn voor consumptie door coeliakie patiënten.

In **hoofdstuk 4** onderzoeken we de veiligheid van haver voor consumptie door coeliakie patiënten. We hebben bevestigd dat zonder uitzondering alle commercieel verkrijgbare haver verontreinigd is met andere granen en dus onveilig voor consumptie door coeliakiepatiënten. Bovendien vonden we dat er variabiliteit bestaat in het niveau van de T-cel stimulerende peptiden tussen havervariëteiten. Dit biedt de mogelijkheid om havervariëteiten te selecteren en/of te ontwikkelen die geen schadelijke glutenachtige eiwitten bevatten.

In **hoofdstuk 5** bepaalden we het potentieel van AN-PEP, een prolyl-endoprotease geproduceerd door het micro-organisme *Aspergillus niger*, om gluten af te breken in een kunstmatig maagdarmkanaal systeem. Het enzym bleek zeer efficiënt te zijn in het afbreken van alle toxische gluten epitopen, zelfs als een complexe maaltijd was ingevoerd. Deze "in vitro" onderzoeken rechtvaardigen nu een klinische studie om de veiligheid en effectiviteit van het enzym te bepalen. Dit kan mogelijk leiden tot een enzympil die patiënten kunnen gebruiken om gluten consumptie mogelijk te maken.

In **hoofdstuk 6** bediscussieer ik hoe deze resultaten kunnen leiden tot nieuwe behandelingsmethoden en nieuwe voedingsmiddelen in de nabije toekomst.

List of publications

LIST OF PUBLICATIONS

- Stepniak D., Spaenij-Dekking L., Mitea C., Moester M., de Ru A., Baak-Pablo R., van Veelen P. Edens L., Koning F. – *Highly efficient gluten degradation with a newly identified prolyl endoprotease: implications for celiac disease.* Am J Physiol Gastrointest Liver Physiol 2006 Oct;291(4):G621-9

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- Dekking L., Mitea C., Stepniak D., Baak-Pablo R., Kooy-Winkelaar Y., Edens L., Koning F. – *Detection and detoxification of gluten*. Proceedings of the 21st Meeting Working group on Prolamin Analysis and Toxicity, Trieste Italy, 2006

Curriculum vitae

CURRICULUM VITAE

The author of this thesis, Doina Cristina Mitea was born in Cluj-Napoca, Romania on the 28th of March 1978. She attended secondary school at the "Tiberiu Popoviciu" Computer Science High School in Cluj-Napoca, where she got her Romanian Baccalaureate diploma in June 1997. From 1998 she studied Medicine at the "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca. During her medical study she performed a 3-months research project at the department of Immunohematology and Blood Transfusion of the Leiden University Medical Center, under the supervision of Prof. dr. Frits Koning and dr. Liesbeth Dekking, investigating the molecular basis of celiac disease. After receiving her MD diploma in September 2004, she returned in the group of Prof. dr. Frits Koning to carry out the present PhD research project that she completed in April 2009. The results of her research led to scientific publications, poster and oral presentations during more international congresses and symposia. In May 2009 she started a 4-year training program in the Leiden University Medical Center to become a specialist in nuclear medicine.

Abbreviations

ABBREVIATIONS

Aa	amino acid;
ACN	acetonitrile
AN-PEP	*Aspergillus niger* prolyl endoprotease
BSA	bovine serum albumin
CD	celiac disease
cpm	counts per minute
ELISA	enzyme-linked immunosorbent assay
GI	gastrointestinal
Glt	glutenin
Glia	gliadin
HMW	high molecular weight
HLA	Human leukocyte antigen
IMDM	Iscove's modified Dulbecco's media
LMW	low molecular weight
mAb	monoclonal antibody
NMP	N-methyl-2-pyrrolidone
PEP	prolyl endopeptidase
PVDF	Polyvinylidene fluoride
PBMC	peripheral blood mononuclear cell
RT	room temperature
Rp HPLC	reversed phase high performance liquid chromatography
SDS-PAGE	sodiumdodecyl sulphate poly-acrylamide gel electrophoresis
TIM	TNO gastrointestinal model
tTG;	tissue transglutaminase
TTd;	tetanus toxoid

Dankwoord

DANKWOORD

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